PHD

A mutant citrate synthase from E. coli

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A MUTANT CITRATE SYNTHASE FROM *E.coli*

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ABBREVIATIONS

Most of the abbreviations used in this thesis are those recommended in the Biochemical Society publication "Policy of the Journal and Instructions to Authors" (1985) (*Biochem. J.* **225**, 1-26.)

<u>Non-Standard Abbreviations</u>

BSA: bovine serum albumin

CS: citrate synthase

CsCl: caesium chloride

DTNB: 5,5' dithiobis-(2-nitrobenzoic acid)

dNTP: deoxyribonucleoside triphosphate

EMS: ethyl methanesulphonate

HAc: acetic acid

IPTG: isopropylthio-β-D-galactoside

KOAc: potassium acetate

LB: Luria-Bertani medium

LDH: lactate dehydrogenase

MDH: malate dehydrogenase

NaOAc: sodium acetate

OAA: oxaloacetate

1x SSC: 150mM NaCl, 15mM Na citrate, pH 7.0

Taq: *Thermus aquaticus*

TBE: 0.09M Tris-borate pH 8.0, 2mM EDTA

TE: 10mM Tris/HCl pH 8.0, 1mM EDTA

TEA: triethanolamine

X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CONTENTS

SUMMARY

1. By mutagenesis, a strain of *Escherichia coli* is created that produces a citrate synthase with markedly different properties when compared with the wild type enzyme.

2. The citrate synthase gene, *glt*A, from this mutant organism is cloned and sequenced together with the *glt*A gene from its parent organism and a wild-type strain of *E.coli.* Sequence analysis reveals a single point mutation within the mutant *glt*A and the same base substitution is observed in the *glt*A gene of the parent organism. The implications of this substitution are discussed.

3. A polyclonal antibody is raised against the wild-type *E.coli* citrate synthase and shows no cross-reactivity with the mutant citrate synthase.

4. The mutant enzyme is purified to near homogeneity and the N-terminus is sequenced. Comparison with protein-data banks does not reveal any homologous proteins. The possible identity of the mutant protein is discussed.

1. INTRODUCTION

1.1 Role of Citrate Synthase in the Citric Acid Cycle

Although the idea of a cyclic pathway as the terminal stage of cellular respiration in animals was proposed by Krebs and Johnson in 1937, it was not until 1950 that the last piece of the puzzle, namely the formation of citrate from oxaloacetate (OAA) and pyruvate, was solved. Stern and colleagues (1950) showed that 'active acetate', later identified as acetyl-CoA (Lynen & Reichert, 1951), was formed from pyruvate and that this combined with OAA by the action of 'condensing enzyme', now known as citrate synthase [EC 4.1.3.7], to form citrate:

$$\text{oxaloacetate} + \text{acetyl CoA} + H_2O \rightarrow \text{citrate} + \text{CoASH} + H^+$$

The citric acid cycle, shown in Fig. 1.1, is the final stage of oxidation of all major foodstuffs in the vast majority of living organisms and has a dual function in being responsible for the production of both energy, as ATP, and the biosynthetic precursors for cell components (Krebs *et al.*, 1952; Roberts *et al.*, 1953)

Citrate synthase (CS) is often regarded as the 'first' enzyme in the citric acid cycle, as it is by the formation of citrate that carbon atoms enter the cycle to be oxidised to CO_2 and to form reducing equivalents. Indeed CS is the only enzyme in the citric acid cycle that is involved in the formation of carbon-carbon bonds. CS is responsible for supplying carbon atoms to the cycle and has been proposed as the key regulating enzyme of the pathway, since intermediates of the cycle do not accumulate (Krebs & Lowenstein, 1960). Further evidence to support this has been provided by digital

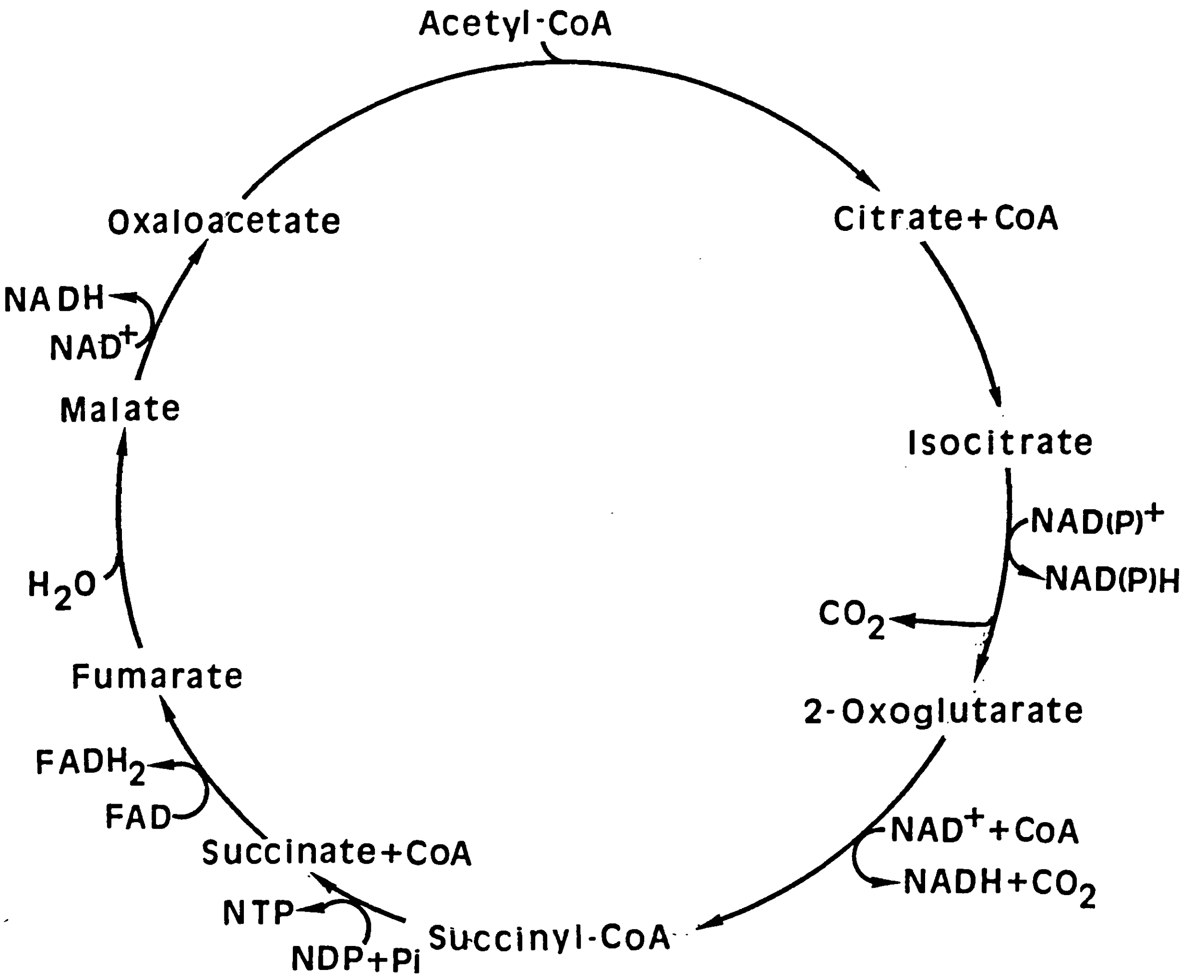


Fig. 1.1. The Citric Acid Cycle

computer simulation of control of the citric acid cycle in rat heart (McMinn & Ottaway, 1976).

In eukaryotic cells CS occurs almost exclusively in the mitochondria, although both a mitochondrial and a peroxisomal form of the enzyme have been identified in yeast (Duntze *et al.*, 1969; Wales *et al.*, 1980) and, in germinating plants, CS is found in both the mitochondria and the glyoxysomes (Breidenbach & Beevers, 1967; Cooper & Beevers, 1969).

1.2 Oligomeric Structure

The central role of CS in metabolism has, therefore, generated much interest and consequently, CS has been purified from a great number of sources, though the pig heart and *E.coli* forms of the enzyme have been the most extensively studied. The amino acid sequences of CS have been determined directly from pig kidney (Evans *et al.*, 1988) and heart (Bloxham *et al.*, 1982) and *E.coli* (Bhayana & Duckworth, 1984) and inferred from DNA sequences of the gene encoding the enzyme, from pig heart (Bloxham *et al.*, 1981), the Gram-negative organisms *E.coli*, (Guest, 1981; Ner *et al.*, 1983), *Pseudomonas aeruginosa* (Donald *et al.*, 1989), *Acinetobacter anitritum* (Donald & Duckworth, 1987) and *Acetobacter aceti* (Fukaya *et al.*, 1990) the yeast *Saccharomyces cerevisiae* (Suissa *et al.*, 1984), the parasitic bacterium *Rickettsia prowazekii* (Wood *et al.*, 1987), the plant *Arabidopsis thaliana* (Ungar *et al.*, 1989), and the archaebacterium *Thermoplasma acidophilum* (Sutherland *et al.*, 1990).

Multiple alignment of these sequences (Sutherland *et al.*, 1990) has shown high sequence identity between the eukaryotic and plant enzymes (48-60%) with an even higher identity existing between the members of Gram-negative bacteria (57-70%).

The pig and chicken heart CSs have been analysed by X-ray crystallography (Remington *et al.*, 1982; Wiegand *et al.*, 1984) revealing that the 437 amino acid residues of each subunit of the pig heart enzyme are arranged as 20 α-helices and a small segment of β-sheet. The crystal stucture of CS from *E.coli* has also been determined, but only at low resolution (Rubin *et al.*, 1983). Using sequence alignment data for pig heart and *E.coli* CSs, Henneke has generated, by molecular modelling, a three dimensional structure for the monomeric unit of *E.coli* CS (Henneke, C.M. *et al.,* unpublished data).

1.3 Regulation

1.3.1 Isosteric Inhibition by Nucleotides

When Hathaway and Atkinson demonstrated in 1965 that CS from yeast was inhibited *in vitro* by ATP, it was postulated that this was a physiological feedback mechanism, as CS is the 'first' enzyme in an ATP producing cycle. ATP was shown to be most effective as an inhibitor when concentrations of ADP and AMP were low, leading Atkinson (1968) to develop the concept of energy charge, in which the *relative* rather than absolute concentrations of the three nucleotides are of metabolic importance.

Since then, a similar inhibition of CSs from a variety of organisms has been demonstrated (Weitzman & Danson, 1976). However, the effect is reduced in the presence of low concentrations (1.5 mM) of Mg^{2+} ions (Kosicki & Lee, 1966) and in *in situ* studies on permeabilised yeast cells (Weitzman & Hewson, 1973) and rat liver mitochondria (Matlib *et al.*, 1978). Moreover, ATP inhibition of CS has been demonstrated in organisms in which the citric acid cycle plays a biosynthetic rather than an energy

Therefore, the suggestion that ATP acts as an *in vivo* regulator of CS is questionable. Nevertheless, the inhibition of the enzyme by ATP has been shown to be competitive with respect to acetyl-CoA (Harford & Weitzman, 1975; Weitzman, 1981) and as they are structurally similar it has been suggested that they bind at the same site (Kosicki & Lee, 1966; Srere *et al.*, 1973; Harford & Weitzman, 1975).

Nicotinamide nucleotides have also been shown to act as weak inhibitors of ATP sensitive CSs (Lee & Kosicki, 1967). Like ATP they share structural similarities with acetyl-CoA and have been shown to compete for the same site on the enzyme (Weitzman, 1981).

1.3.2 Allosteric Inhibition by NADH

In contrast to ATP, NADH, also an 'end product' of the cycle, has gained more favour as a specific feedback regulator of CS. The *E.coli* enzyme, although only weakly inhibited by ATP, is very sensitive to NADH and is inhibited in an allosteric manner (Weitzman, 1966a; Faloona & Srere, 1969). This inhibition is specific to NADH as no other nucleotides inhibit *E.coli* CS. Other Gram-negative bacterial CSs were studied and these too were inhibited specifically by NADH (Weitzman & Jones, 1968). Conversely, eukaryotes and Gram-positive bacteria were shown to produce CSs that were unaffected by NADH. Moreover, the Gram-negative bacteria could be further divided into two groups, as the NADH inhibition of some (e.g. *A.calcoaceticus*) was reversed by AMP. Further examination revealed that these were strictly aerobic bacteria. These organisms rely solely on the citric acid cycle for energy production and, therefore, it makes sense for AMP, an indicator of low energy levels, to regulate a key enzyme in the cycle.

1.3.3 Biosynthetic Control

As the citric acid cycle is responsible not only for the production of energy but also for the production of biosynthetic precursors, it would seem appropriate that these substances also exert some regulatory control on CS. 2-oxoglutarate and succinyl-CoA, both produced by the citric acid cycle, are used for amino acid and porphyrin biosynthesis respectively and have been reported to inhibit CSs from certain organisms.

(i) Inhibition by 2-oxoglutarate

The inhibition of CS by 2-oxoglutarate was first reported by Wright *et al.* (1967). This prompted Weitzman and Dunmore (1969) to examine a range of organisms and they found that only CSs from facultatively anaerobic Gram-negative bacteria were inhibited by 2-oxoglutarate, whereas aerobic Gram-negative bacteria, Gram-positive bacteria and eukaryotes were unaffected. This is most likely due to the fact that facultative anaerobes can produce energy by carbohydrate fermentation and under such conditions the enzyme 2-oxoglutarate dehydrogenase is absent. Thus, a branched non-cyclic pathway operates to provide the biosynthetic precursors 2-oxoglutarate and succinyl-CoA (Amarasingham & Davis, 1965) (Fig. 1.2). Further investigations of facultatively autotrophic *Thiobacilli* sp. (Taylor, 1970), Gram-positive facultative anaerobes (Gottschalk & Dittbrenner, 1970) and strict anaerobes (Tanaka & Hanson, 1975) have revealed that inhibition by 2-oxoglutarate is not restricted to Gram-negative facultative anaerobes, but is a feature of all bacteria that lack 2-oxoglutarate dehydrogenase and hence have 2-oxoglutarate as a biosynthetic end product of CS activity.

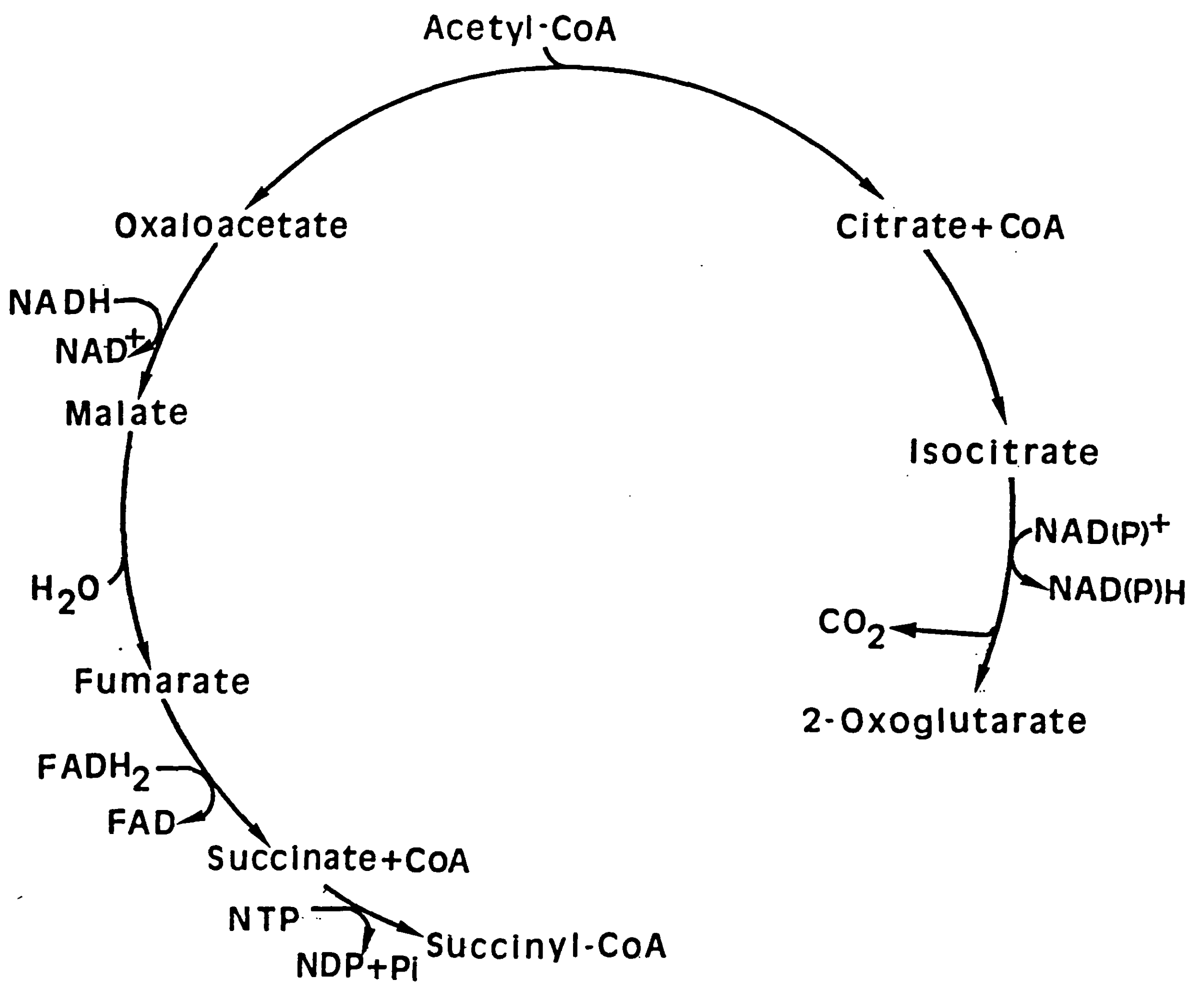


Fig. 1.2. Branched Non-cyclic Pathway Operating in Facultative Anaerobes

ii) Inhibition by 2-oxoglutarate and Succinyl-CoA

An additional feedback control mechanism is found in the Gram-negative cyanobacteria. Like facultative anaerobes, cyanobacteria lack 2-oxoglutarate dehydrogenase and do not use the citric acid cycle for energy production. Therefore, their CSs are inhibited by 2-oxoglutarate (Lucas & Weitzman, 1977) but not by NADH (Taylor, 1973) However, unlike the situation in *E.coli*, succinyl-CoA is also formed from citrate via the glyoxylate cycle (Pearce *et al.*, 1969) (Fig. 1.3). Succinyl-CoA is, therefore, an end product of the initial reaction catalysed by CS in cyanobacteria and has been found, in addition to 2-oxoglutarate, to inhibit CS in these organisms (Lucas & Weitzman, 1977). This inhibition by succinyl-CoA is specific to cyanobacteria and would appear to reflect the unique metabolic pathway displayed by these organisms.

1.4 Molecular Size

An examination by Weitzman and Dunmore (1969) of the enzyme from a variety of sources revealed that CSs can be divided into two groups according to their molecular sizes. Those of eukaryotes and Gram-positive bacteria are termed 'small', having a M_r~100 000, whilst Gram-negative bacteria produce a 'large' enzyme of M_r ~250 000. Additional CSs have subsequently been analysed and these studies have further confirmed this distribution of molecular sizes.

The 'small' enzyme was shown to be a dimer of identical subunits of M_r~50 000 in eukaryotes (Srere,1972) and was later, also shown to be the case in Gram-positive bacteria (Weitzman & Danson, 1976) and Archaebacteria (Danson *et al.*, 1985). The elucidation of the subunit composition of the 'large' enzyme was, however, not so straightforward.

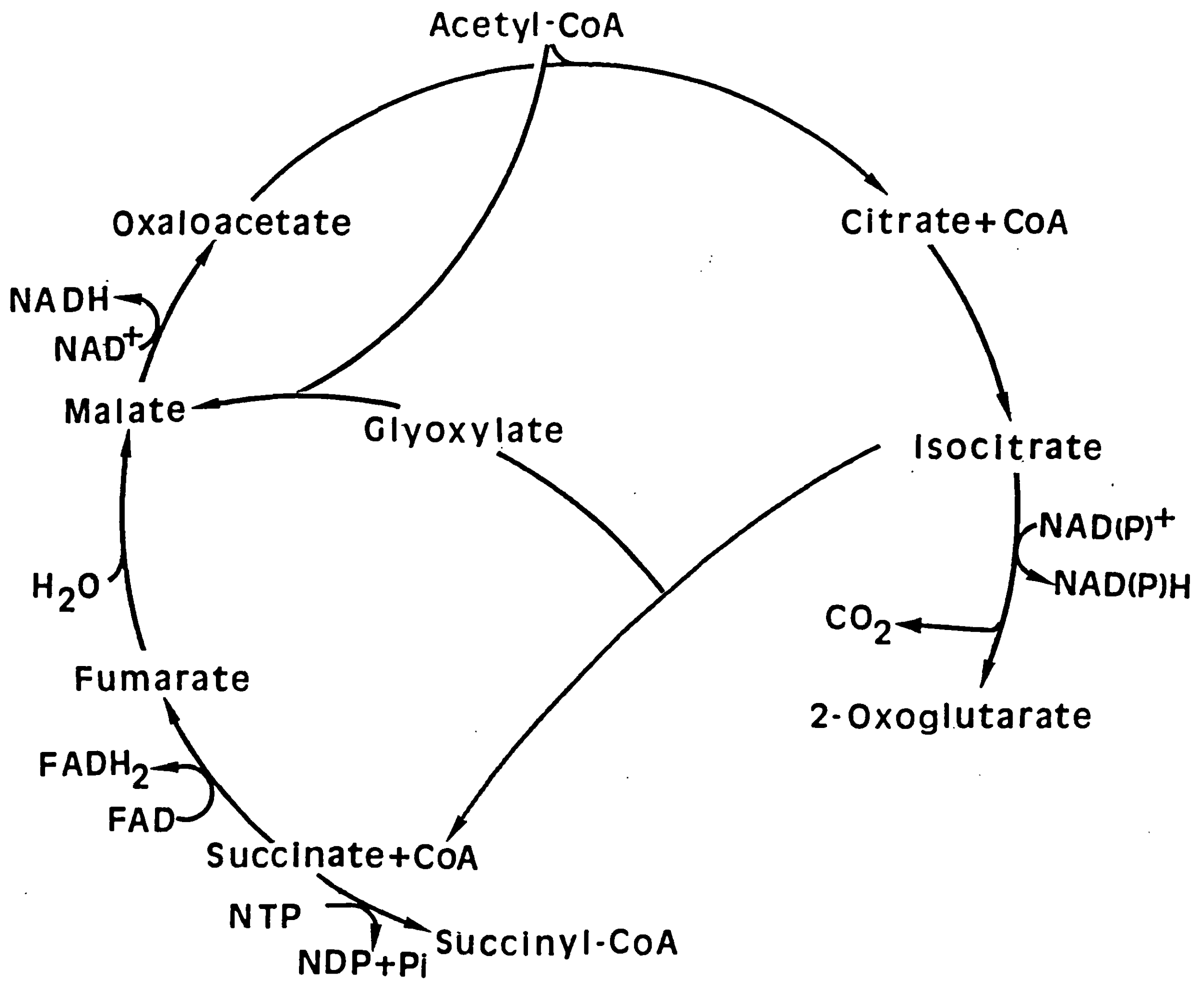


Fig. 1.3. The Glyoxylate Cycle

Originally, it was reported that the enzyme from *E.coli* was a tetramer (Wright & Sanwal, 1971), each subunit being identical and having a M_r~62 000. Later work by Tong and Duckworth (1975), using a combination of equilibrium ultracentrifugation and cross-linking with dimethyl suberimidate followed by SDS PAGE, showed the *E.coli* enzyme to be a hexamer of subunit M_r~47 000. A predominance of even-numbered species on analysis of the cross-linked enzyme, together with ultracentrifugation results showing that a dimer is the only species present in dilute buffer at pH 9.0 and is one of two species present in 50 mM KCl, indicated that a dimeric arrangement of the subunits of the *E.coli* enzyme is exceptionally stable and may be equivalent to the eukaryotic enzyme. Proteolysis studies of CS from *E.coli* (Else *et al.*, 1988) and *A.calcoaceticus* (Mitchell & Weitzman, 1983) have indicated that the hexameric structure is arranged as a trimer of dimers. Thus, the dimer is the basic catalytic unit of the enzyme from eukaryotes, Archaebacteria, Gram-positive *and* Gram-negative bacteria.

1.5 Correlation of Oligomeric Properties and Regulatory Sensitivities

In summary, a pattern of diversity exists with respect to molecular size and regulatory function of CS. Eukaryotic and Gram-positive bacterial CSs are 'small' dimeric proteins, whereas Gram-negative bacterial CS is a 'large' hexameric protein. The two proteins differ not only in size but are also regulated by different effectors. The 'small' proteins are probably regulated isosterically by ATP and the 'large' protein allosterically by NADH. Inhibition of CS by NADH is specific and is reversed by AMP in strictly aerobic organisms. In addition, CSs from facultative anaerobes are inhibited by 2-oxoglutarate probably as a result of their mainly biosynthetic role in these organisms (Table 1.1).

	EUKARYOTES	EUBACTERIA Gram +ve	Gram -ve
MOLECULAR WEIGHT (kDa)	100 000	90 000	270 000
SUBUNIT COMPOSITION	2N	2N	6N
INHIBITORS	ATP	ATP	NADH

Table 1.1. Diversity of CSs

1.6 Exceptions and Extensions to the Pattern of Enzyme Diversity

Although the majority of CSs studied so far follow the pattern above, there are some, nevertheless, that do not. Several Gram-negative bacteria have been identified that produce CSs that although characteristically of the 'large' type are insensitive to NADH (Swissa & Benziman, 1976; Weitzman, 1981).

In addition, Phibbs and Winkler (1982) have reported that the enzyme from the Gram-negative intracellular parasite *R.prowazekii* has unusual characteristics being of the 'small' type enzyme but not inhibited by ATP.

This review has concentrated on eukaryotic and eubacterial CSs yet a third group of organisms have been identified: the Archaebacteria (Olsen & Woese, 1989). Although only a handful have been studied so far, they have all been shown to produce 'small' NADH insensitive CSs (Cazzulo, 1973; Danson *et al.*, 1985: Sutherland *et al.*, 1990).These organisms are generally found in extreme environments such as high temperature and high salt concentrations and it may be that the dimer is more able to maintain its native structure than the 'large' hexameric form under these disruptive conditions. Moreover, inhibition of eubacterial CS by NADH has been shown to be lost in increased salt concentrations (Weitzman, 1966b; Massarini *et al.*, 1976) and, therefore, this regulatory function would be ineffective in the organisms natural environment.

1.7 Multiple Forms of the Enzyme

Whilst the majority of organisms contain only one form of CS a few organisms have been identified that produce multiple forms of the enzyme. Mitochondrial and glyoxysomal CSs have been isolated in plants (Breidenbach & Beevers, 1967; Cooper & Beevers, 1969) and both a mitochondrial and a peroxisomal enzyme have been found in the yeast *S.cerevisiae* (Duntze *et al.*, 1969; Wales *et al.*, 1980). In both cases the enzymes appear to be all of the 'small' type. The plant enzymes display different kinetic properties (Axelrod & Beevers, 1972; Barbareschi *et al.*, 1974) and can be separated by chromatography (Barbareschi *et al.*, 1974). Yet despite these differences both the glyoxisomal and mitochondrial CSs cross-react with anti-glyoxisomal CS antibodies (Huang *et al.* 1974). The two yeast enzymes are encoded by distinct but highly homologous (75% identity) genes (Kim *et al.*, 1986; Rickey & Lewin, 1986; Rosenkrantz *et al.*, 1986) but unlike the plant enzymes, antibodies raised against yeast mitochondrial CS do not cross-react with the peroxisomal form (Rickey & Lewin, 1986) although there is a high degree of homology in their amino acid sequences. It has been proposed that the enzymes also differ in their metabolic function: the non-mitochondrial forms participating in the glyoxylate cycle (Breidenbach & Beevers, 1967; Cooper & Beevers, 1969; Kispal *et al.*, 1989; Lewin *et al.*, 1990).

Whereas these CSs are all of one type, a different situation has been found in various *Pseudomonas* species. It has been reported that these species possess two types of CS: a 'large' NADH sensitive enzyme and a 'small' NADH insensitive enzyme (Solomon & Weitzman, 1983; Mitchell & Weitzman, 1986). The relative proportions of each vary with the phase of growth of the organism (Solomon & Weitzman, 1983). The two enzymes do not interconvert and so, unlike previous studies (Massarini & Cazzulo, 1975; Higa *et al.*, 1978), are not different forms of the same enzyme. However, a recent study observed only the 'large' NADH sensitive form, as judged by gel filtration, antibody binding and molecular biology techniques (Donald *et al.*, 1989).

1.8 Kinetic Mechanism

Kinetic and binding studies have established that CS acts by an ordered mechanism involving the formation of a two substrate ternary complex (Shepherd & Garland, 1969; Johansson *et al.*, 1973; Matsuoka & Srere, 1973). Oxaloacetate binds first, increasing the binding constant for acetyl-CoA by at least 20 fold (Johansson & Petersson, 1974). Binary complex formation with acetyl-CoA has also been shown to take place at high acetyl-CoA concentrations but this substantially decreases the on-rate for OAA, indicating substrate inhibition (Johansson & Petersson, 1977). The rate-limiting step during catalysis appears to be the conversion of the ternary enzyme·substrate complex into a ternary enzyme·product complex (Bové *et al.*, 1959; Kosicki & Srere, 1961).

CS is highly specific towards its substrates as only acetyl-CoA (Stern *et al.*, 1951), fluoroacetyl-CoA (Brady, 1955), OAA (Stern *et al.*, 1951) and fluorooxaloacetate (Fanshier *et al.*, 1964) have been shown to be substrates for the condensation reaction, although a number of acetyl-CoA analogues have been shown to bind to the enzyme (Chase *et al.*, 1966; Stern, 1961; Weidman *et al.*, 1971).

Citryl-CoA was shown to inhibit eukaryotic CS (Srere & Kosicki, 1963), and it was postulated that citryl-CoA was an intermediate of the reaction. Eggerer and Remberger (1963) were the first to show that citryl-CoA could indeed be hydrolysed by the enzyme, a result that was later confirmed by Srere (1963) and more recently by Pettersson (Pettersson *et al.*, 1989).

The stereochemistry of the reaction catalysed by CS has been elucidated showing that the acetyl group of acetyl-CoA adds to the si-face of the keto moiety of OAA (Bové *et al.*, 1959; Englard, 1959; Hanson & Rose,

1963) with a concomitant inversion of the configuration of the methyl hydrogens (Eggerer *et al.*, 1970; Retéy *et al.*, 1970; Klinman & Rose, 1971; Lenz *et al.*, 1971).

The overall reaction (Eggerer, 1965) can be divided into three partial reactions, namely i) generation of a carbanion on acetyl-CoA by removal of a proton, ii) nucleophillic attack of the carbanion form of acetyl-CoA to form citryl-CoA and iii) hydrolysis of citryl-CoA to produce citrate and CoA.

1.9 Conformational Changes During Catalysis

Early studies using uv spectra, kinetics and spin labels provided a wealth of information suggesting that a change in conformation of CS occurred on binding its substrates (Srere,1966; Rowe & Weitzman, 1969; Weidman *et al.*, 1973; Johansson & Pettersson, 1979; Bayer *et al.*, 1981).

Crystal structure analysis has also provided considerable insight into the structural and functional properties of the enzyme. Remington (Remington *et al.*, 1982) observed two forms of the enzyme from pig heart, a closed (monoclinic) crystal in the presence of citrate and CoA and an open (tetragonal) crystal representing the free enzyme. Each subunit was shown to be composed of a large and a small domain connected by a 'hinge' region. The two subunits were shown to be extensively interdigitated with residues from one subunit contributing to the active site of the other. Thus, the two subunits are dependent on each other for complete catalytic activity and would, therefore, explain the importance of the dimeric unit as previously described (1.2). The active site was shown to lie in a deep cleft between the two domains of each subunit and it was proposed that binding of OAA induces the change in conformation from the open to closed form, bringing about a change in configuration of active site residues (Lesk &

Chothia, 1984) in order that catalysis may proceed. Wiegand *et al.* (1984) described a third crystal structure of the pig heart enzyme in the presence of OAA and S-acetonyl-CoA (a potent inhibitor of CS). This third form was described as 'nearly closed' with considerable re-arrangement within the domains compared with the two previously described forms. Wiegand and Remington (1986) proposed that OAA binds to the open form of the enzyme inducing the closed (or nearly closed) form. This form polarises and orientates OAA and provides a binding site for acetyl-CoA. As acetyl-CoA enters the active site a proton is removed from the methyl group and condensation occurs, resulting in the inversion of the configuration of the hydrogens of the methyl group. The appearance of citryl-CoA on the enzyme induces the closed form and subsequent hydrolysis releases the products citrate and CoA. Thus, the nearly closed form and closed form might reflect the different catalytic states of the enzyme, namely, condensation and hydrolysis (Bayer *et al.*, 1981; Löhlein-Werhahn *et al.*, 1983; Lill *et al.*, 1984; Lill *et al.*, 1987; Löhlein-Werhahn *et al.*, 1988). However, this has recently been questioned by studies using fluorescent probes (Kollman-Koch & Eggerer, 1989) and crystallography (Karpusas *et al.*, 1990, Karpusas *et al.*, 1991: Liao *et al.*, 1991). Karpusas (Karpusas *et al.*, 1990) has crystallised a ternary enzyme·OAA·carboxymethyl-CoA complex, refined at 1.9 Å, from chicken heart that they suggest is capable of catalysing the whole reaction and have shown that L-malate, a structural analogue of OAA, binds to the closed conformation (Karpusas *et al.*, 1991) . Recently Liao *et al.* (1991) have suggested that the closed and open forms are in equilibrium in solution and that if ligands with large binding constant are present then the equilibrium is driven towards the closed conformation.

1.10 Catalytic Mechanism

Early work investigating the residues responsible for catalysis made use of a variety of chemical modifiers, although the majority of work has concentrated on sulphydryl modifiers such as DTNB, iodoacetate and 2-nitro-5-thiobenzoic acid. Modification of sulphydryl groups produced different responses from organism to organism. CSs from eukaryotes have generally been shown to be totally insensitive to sulphydryl modification (Srere *et al.*, 1963; Srere, 1965; Weitzman & Danson, 1976). Some plants have been shown to be sensitive to sulphydryl modification (Srere *et al.*, 1971; Greenblatt & Sarkissian, 1973) whereas others have been shown to be totally insensitive (Srere *et al.*, 1971). The same diversity of response was observed in CSs from Gram-positive and Gram-negative bacteria (Dittbrenner *et al.*, 1969; O'Brien & Stern, 1969; Danson & Weitzman, 1973; Weitzman & Danson, 1976; Danson & Weitzman, 1977; Talgoy & Duckworth, 1979). However, in Gram-negative bacteria where inactivation of the enzyme by sulphydryl modification was observed there was also a desensitisation of the enzyme to NADH. This implied that cysteine residues are involved in this regulatory function. This cysteine residue has recently been identified as Cys 206 in *E.coli* which is located near the interface between the two subunit domains (Duckworth *et al.*, 1987) and may be essential in allowing the necessary movement of the domains to orientate the active site. Likewise the binding of NADH to a sulphydryl group in this position would interfere with the movement of the two domains thus preventing catalysis and would explain the inhibitory effect of NADH. Examination of the nucleotide sequences published to date, reveals that in those organisms sensitive to sulphydryl modification, a cysteine residue is present at the equivalent position (Table 1.2).

Photo-oxidation studies have also been used to study the catalytic mechanism of CS and have implicated the importance of several amino acids. Histidine residues were suggested as being involved in the enzymatic activity and the inhibition by 2-oxoglutarate (Danson & Weitzman, 1973; Weitzman *et al.*, 1974; Måhlén, 1975; Weitzman & Danson, 1976). Arginine and tryptophan residues (Danson & Weitzman, 1973; Måhlén, 1975; Weitzman & Danson, 1976) also appear to play some part in catalysis. Furthermore, using proteolysis Bloxham and colleagues (1980) proposed that the binding site for OAA and citrate was in a region corresponding to residues 336-337.

Crystallographic studies of the enzyme from pig heart and chicken heart have verified these findings, showing that indeed three histidine (238, 274, 320) and three arginine residues (329, 401, 421) are in contact with citrate and OAA and three arginine residues (46, 164, 324) and one lysine residue (368) form salt bridges with CoA at the active site (Remington *et al.*, 1982; Wiegand *et al.*, 1984; Karpusas *et al.*, 1990; Karpusas *et al.*, 1991). The majority of nucleotide sequences analysed so far have all these residues conserved in equivalent positions although Arg 46 and Arg 164 are less well conserved in Gram-negative organisms (Ner *et al.*, 1983; Donald & Duckworth, 1987; Wood *et al.*, 1987) (Table 1.2). It has been suggested (Ner *et al.*, 1983) that lack of these residues is associated with a high K_m for acetyl-CoA, but this has been questioned by Wood (Wood *et al.*, 1987) as *R.prowazekii* possesses neither residue yet has a K_m for acetyl-CoA equivalent to that of pig heart.

Three amino acids were identified as being potential key residues in catalysis: His 274, His 320 and Asp 375 and these have been shown, by multiple alignment, to be conserved throughout all CS sequences analysed so far (Donald *et al.*, 1989; Henneke *et al.*, 1989; Unger *et al.*, 1989; Sutherland *et al.*, 1990) (Table 1.2). It has been proposed (Remington *et al.*, 1982; Karpusas *et al.*, 1990) that His 274 and Asp 375 act as proton acceptors and donors to the acetyl and methyl groups of acetyl-CoA whilst His 320 is in an ideal position to protonate the carbonyl oxygen of OAA. It has also been suggested that Asp 375 protonates the intermediate citryl-CoA causing the release of CoA (Fig. 1.4).

Site directed mutagenesis has further helped to identify the residues important for ligand binding and catalysis. Changing His 229 of *E.coli* CS (equivalent to His 238 of pig heart) or His 226 of *E.coli* CS to Glu resulted in a reduced affinity for OAA and 2-oxoglutarate which was more pronounced for the His 229 mutation (Anderson & Duckworth, 1988), confirming the proposal that this is one of the substrate binding sites (Remington *et al.*, 1982). Evans *et al.* (1989) and Alter *et al.* (1990) have clearly shown that changing Asp 375 of pig heart CS to amino acids that do not have a dissociable proton (i.e Asn, Gln, and Gly) renders the enzyme virtually inactive. Handford *et al.* (1988) showed a similar inactivation when Asp 362 of *E.coli* CS (equivalent to Asp 375 of pig heart CS) was replaced with Gly. Asp 362 was shown to be essential not only for catalysis but also for the binding of acetyl-CoA but not OAA. In addition replacing His 274 of pig heart CS with Gly or Arg (Evans *et al.*, 1989; Alter *et al.*, 1990) also reduced the enzyme's activity. These results are summarised in Table 1.3.

1.11 Mutant Forms of Citrate Synthase

Chemical modification, X-ray crystallography and site directed mutagenesis have provided us with a considerable insight into the structure and regulatory properties of CSs. However, there is still virtually nothing

ligand bound in crystal structure	Pig Heart	E.coli	A.anitratum	P.aeruginosa	S.cerevisiae 1	S.cerevisiae 2	T.acidophilum	R.prowasekii	A.thaliana
	arg 329	314	315	314	368	348	271	319	359
	arg 401	387	388	387	440	420	344	394	429
	arg 421	407	408	407	460	440	364	415	449
Citrate	his 238	229	226	229	277	254	187	234	268
	his 274	264	267	264			222	269	304
	his 320	305	306	305	313	393	262	310	350
	asp 375	362	363	362	414	394	317	369	403
CoA 3'-phosphate	arg 164	157	159	157		184	121	-	lys 235
	arg 46	-	-	-	86	66	lys 8	-	arg 84
CoA 5'-diphosphate	arg 324	lys309	lys310	lys309	363	343	lys 266	lys314	arg 354
	Me_3lys 368	lys356	arg356	arg355	407	387		lys363	arg 396
NADH	-	cys206	ala208	cys206	l ys255	235cys		cys211	

Table 1.2 Conserved Residues in CSs

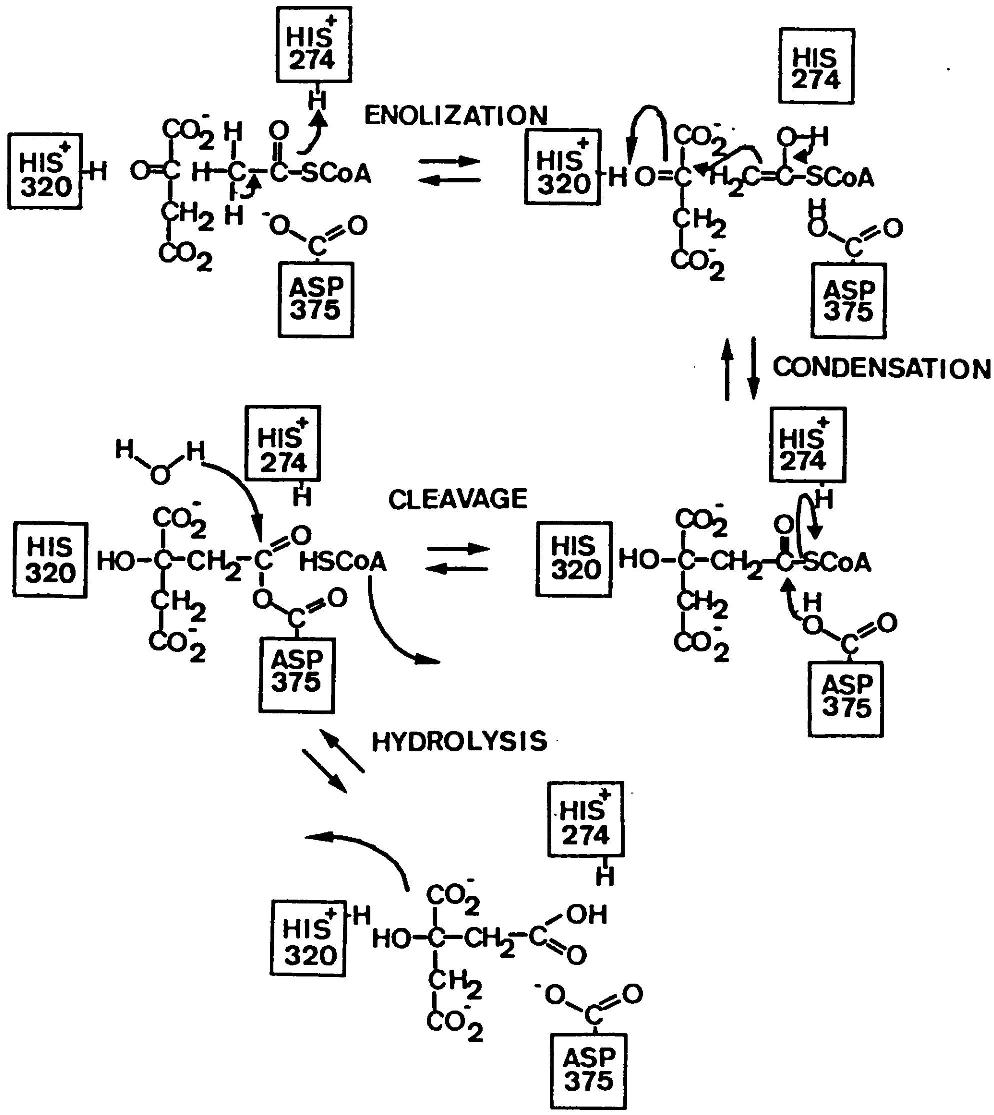


Fig. 1.4. Proposed Mechanism of Citrate Synthase Reaction

	NON MUTANT	MUTANT	EFFECT
PIG HEART	ASP 375	ASN GLN GLY	INACTIVATION
	HIS 274	GLY ARG	ACTIVITY DECREASED BY 10^3
E.coli	ASP 362	GLY	INACTIVATION
	HIS 229 HIS 226	GLU	REDUCED SUBSTRATE AFFINITY

Table 1.3. Site directed Mutagenesis of CS

known regarding subunit interactions of the enzyme and how these interactions effect the regulation of the enzyme, bearing in mind that only 'large' hexameric CSs are regulated by NADH. Bacterial mutants deficient in CS have been used in many studies to investigate the physiological role of the enzyme (Gilvarg & Davis, 1956; Ashworth *et al.*, 1965; Carls & Hanson, 1971; Lakshmi & Helling, 1976; Beatty et al., 1977) and this mutant approach has been used to provide a tool crucial to the attempt to answer these questions regarding subunit interactions. The aim has been to isolate mutant organisms producing variant forms of CS with altered structure and regulation. Investigation of such mutant enzymes may provide information on molecular requirements for the different subunit arrangements and regulatory effectors observed in CSs from various sources.

The strategy used for obtaining mutant forms of the enzyme has been first to produce a CS deficient mutant, to mutate that organism and then to select revertants that have regained CS activity. The regaining of activity would in some cases be a result of second site mutations within the gene leading to the production of a CS with a slightly altered amino acid composition from the parent organism and it was hoped that such altered composition may in some cases lead to altered structural and/or regulatory properties of the enzyme.

Use of such an approach has produced revertants of citrate synthase from *E.coli* (Danson *et al.*, 1979), *A.calcoaceticus* (Weitzman *et al.*, 1978) and *Pseudomonas* (Solomon & Weitzman, 1983) with markedly different properties from the wild type enzyme.

Three different types of revertant have been isolated from *E.coli*. One produced a 'large' enzyme with kinetic and regulatory properties similar to those displayed by the wild type enzyme. A second also produced a 'large' enzyme but unlike the wild type enzyme was not inhibited by 2-oxoglutarate. The third type, however, produced a 'small' enzyme that was not inhibited by 2-oxoglutarate nor NADH and displayed hyperbolic rather than wild type sigmoidal dependences of rate on the concentration of acetyl-CoA and OAA. Such properties more resemble those of the Gram-positive/eukaryotic enzyme than the Gram-negative *E.coli* parent enzyme.

Revertants obtained from *A.calcoaceticus* also displayed a similar array of properties to those from *E.coli*. One class was a 'large'enzyme that, like the wild type enzyme, was inhibited by NADH and reactivated by AMP. The other produced a 'small' enzyme that was insensitive to NADH. Once again it appeared that an enzyme more resembling that from a Gram-positive organism had been produced from a Gram-negative bacterium by genetic modification.

All these mutants are thought to be a result of minor genetic changes as they were produced by mild mutagenic methods and reverted easily to the wild type.

The production of a 'family' of variant enzymes that mimic the diverse forms of naturally-occuring CS have provided us with a valuable tool with which to investigate at the molecular level the array of structures and regulatory properties observed. It is difficult to make any observation regarding structure-function relationships from a study of naturally occurring CSs because evolution may well have introduced amino acid sequences that do not alter the properties of the enzyme. However, such problems are minimised when one investigates variants generated by minor modification of the wild type enzyme.

1.12 Aims of this Work

The aim of this work is, therefore, to create a 'small' mutant CS from *E.coli* as has been done previously (Danson *et al.*, 1979), to clone the gene encoding this enzyme and to sequence the gene in order to identify the amino acid changes that have brought about the altered properties.

2. MATERIALS

2.1 Organisms

The bacterial strains used in this work are listed in Table 2.1A . Individual growth requirements are listed in Table 2.1B. K114 was a gift from Sir Hans Kornberg (Dept of Biochemistry, University of Cambridge, U.K.).

2.2 Chemicals

Tryptone and yeast extract were from Oxoid Ltd, London, U.K; Agar was from Difco Labs, Detroit, U.S.A; CoA and NADH were from Boehringer Mannheim, Germany; DEAE-Sephadex and Sepharose CL6B were from Pharmacia LKB, Milton Keynes, U.K.; Matrex Blue was from Amicon, Stonehouse, Glos.; Protamine sulphate was from Koch-Light, Colnbrook, U.K; CsCl was from Rose Chemicals, London, U.K; Acrylamide, ammonium persulphate, 2-mercaptoethanol, sodium dodecyl sulphate and N,N,N′,N′-tetramethylethylene diamine were from BDH Ltd, Poole, U.K; All radioactive isotopes were from Amersham, Bucks., U.K; All other chemicals were from Sigma Chemical Company Ltd, Poole, U.K.

2.3 Enzymes

Rabbit IgG conjugated to horseradish peroxidase was from Sigma; All restriction endonucleases and DNA modifying enzymes were from Northumbria Biologicals Ltd, Cramlington, U.K; *Taq* polymerase was from Perkin Elmer Cetus, Norwalk, USA. Sequenase sequencing kit was from United States Biochemical Corporation (Cambridge Bioscience, Cambridge, U.K.). All other enzymes were from Boehringer. Multiprime DNA labelling kit was from Amersham.

Table 2.1: Bacterial Strains

A. Genotype

Organism	Genotype
E.coli	
K114	Hfr,*pps,met,thy,glt*A,str^{S}
K114r4	Hfr,*pps,met,thy*
DB1002	*thi*1,*pyr*D36,*gal*K30,*rps*L129
HB101	*sup*E44 *hsd*S20 (r_S^-,m_S^-) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1
TG1	*sup*E *hsd*L5 *thi*L(*lac-pro*AB) F'[*tra*D36 *pro*AB^{+} *lac*I^{q} *lac*ZLM15)

Table 2.1: Bacterial Strains

B. Growth Requirements

Organism	Special Growth Requirements	Comments
E.coli		
K114	methionine, thymine and glutamate	CS^-
K114r4	methionine and thymine	*glt*A revertant producing' small' CS
DB1002	uracil, thiamine and glutamate	contains wild type *glt*A cloned into pBR322
HB101	leucine and proline	
TG1	thiamine	

2.4 Special Preparation of Reagents

2.4.1 Preparation of Acetyl-CoA

Acetyl-CoA was prepared by the method of Stadtman (1957). 10mg of CoA (Boehringer) were dissolved in 1ml water and the solution cooled on ice. 0.2ml of 1M $KHCO_3$ were added to bring the pH of the solution to neutrality. 5μl of acetic anhydride were added and the solution left on ice for at least 10 min. This should give a solution of approximately 7mM acetyl-CoA.

Acetylation was tested by reaction with 0.1mM DTNB, 20mM Tris/HCl pH 8.0 and monitoring at 412nm. Complete acetylation was achieved if no yellow colour developed.

2. 4.2 Equilibration of Phenol

All phenol used was pre-equilibrated to > pH 7.8 to avoid losing the DNA in the organic phase. An equal volume of 0.5M Tris/HCl pH 8.0 was added to the phenol and the mixture stirred on a magnetic stirrer for 15 min. The mixture was allowed to stand until the two phases had separated and then the upper aqueous layer was removed. An equal volume of 0.1M Tris/HCl pH 8.0 was added to the phenol and the mixture stirred as before. The two layers were again separated and the Tris/HCl extractions repeated until the pH of the phenol layer was 8.0 (as measured with pH paper). The equilibrated phenol was store at -20 °C under 100mM Tris/HCl pH 8.0 until required.

2.4.3 RNAase A

RNAase A that was free of DNAase was prepared by the following method. 10mg of RNAase A were dissolved in 1ml 10mM Tris/HCl pH 7.5, 15mM NaCl. The solution was heated to 100 °C for 15 min and then allowed to cool slowly to room temperature. The solution was then dispensed into aliquots and stored at -20 °C until required.

2.4.4 Denatured Salmon sperm DNA

A solution of 10mg salmon sperm DNA/ml water was sheared by passing through a 19-gauge hypodermic needle several times. The solution was stored at -20 °C in small aliquots until required. Just prior to using, the solution was heated for 10 min in a boiling water bath and then chilled quickly on ice.

3. METHODS

3.1 Growth and Maintenance of Bacteria

All bacterial strains were kept at -70 °C.

Growth in liquid media was at 37 °C with shaking.

All media was sterilised by autoclaving prior to use.

Cells were grown routinely in Luria-Bertani (LB) medium (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl per litre).

Minimal media were based on the basal salts medium of Ashworth and Kornberg (1966): 50mM Na/K PO_4 buffer pH 7.2 with 50mM NH_4Cl, 0.18mM $CaCl_2$, 0.018mM $MnSO_4$ and 0.014mM $FeSO_4$.

Appropriate carbon source, sterilised by autoclaving, and any growth requirements, filter sterilised, (Table 2.1B) were added to the minimal medium after autoclaving.

When required, ampicillin was added to the autoclaved medium after cooling to 60 °C.

For solid media 1.5g agar/100ml medium were added.

α-complementation was detected by mixing 50μl of 2% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethylformamide and 10μl of 100mM IPTG (isopropylthio-β-D-galactoside) with the bacterial cells prior to plating out.

3.2 Harvesting of Cells and Preparation of Cell Free Extracts

Cells were harvested by centrifugation at 11 000g for 10 min at 4 °C.

Cell free extracts were prepared by sonication (Ultrasonics 180W sonicator) for 2 min in 15 s bursts with 15 s cooling. When a volume less than 10ml was being sonicated, a 3mm probe was used and sonication carried out at 50W. For volumes greater than 10ml, a 19mm probe was used and sonication carried out at 180W. Cell debris was removed by centrifugation in a microfuge at full speed for 5 min or at 5 000 g for 10 min for large volumes.

3.3 Assay of Citrate Synthase [EC 4.1.3.7]

Citrate synthase was assayed spectrophotometrically at 412nm, 25 °C according to the method of Srere *et al.* (1963). Unless otherwise stated, the assay mixture contained 20mM Tris/HCl pH 8.0, 1mM EDTA, 0.15mM acetyl-CoA (2.4.1), 0.2mM OAA and 0.1mM DTNB (5.5'-dithiobis(2-nitrobenzioc acid). The CoA produced reacts with the DTNB to form a yellow derivative thio-nitrobenzoate ($TNBS^-$). The molar extinction coefficient of $TNBS^-$ was taken to be 13 600 $l.mol^{-1}cm^{-1}$ at pH 8.0 (Ellman, 1959).

3.4 Assay of Lactate Dehydrogenase [EC 1.1.1.27]

Lactate dehydrogenase (LDH) was assayed spectrophotometrically at 25 °C by measuring the oxidation of NADH at 340nm. Unless otherwise stated, the assay mixture contained 20mM Tris/HCl pH 8.0, 1mM EDTA, 0.2mM pyruvate and 0.2mM NADH. The molar extinction coefficient of NADH was taken to be 6 220 $l.mol^{-1}cm^{-1}$ at pH 8.0. Any non-specific NADH oxidation was accounted for by assaying in the absence of pyruvate.

3.5 Assay of Malate Dehydrogenase [EC 1.1.1.37]

Malate dehydrogenase (MDH) was assayed spectrophotometrically at 25 °C by measuring the oxidation of NADH at 340nm. Unless otherwise stated, the assay mixture contained 20mM Tris/HCl pH 8.0, 1mM EDTA, 0.2mM OAA and 0.2mM NADH. The molar extinction coefficient of NADH was taken to be 6 220 $l.mol^{-1}cm^{-1}$ at pH 8.0. Any non-specific NADH oxidation was accounted for by assaying in the absence of OAA.

3.6 Estimation of Protein Concentration

Protein concentration was estimated using the method of Bradford (1976). To 800µl of sample in 20mM Tris/HCl pH 8.0 was added 200µl of BioRad Protein Assay concentrated dye reagent (BioRad Laboratories, Ltd.) and after allowing the colour to develop for 5 min, the absorbance was measured at 595nm. The values obtained were compared with a standard curve of thyroglobulin concentrations.

3.7 Mutagenesis using Ethyl Methanesulphonate (EMS)

Cells from 200ml of an overnight culture in minimal medium were harvested and resuspended in 20ml of 100mM Na/K PO_4 buffer pH 7.0. 200µl of EMS were added and the suspension incubated at 37 °C for 60 min. 1 ml of the cells were collected by centrifugation for 1 min in a microfuge, resuspended in 100µl 100mM Na/K PO_4 buffer and spread onto glucose minimal media plates.

3.8 Molecular Weight Estimation of Citrate Synthase

Size differences of revertant CSs was investigated using a Superose 12 column connected to a Pharmacia LKB FPLC system. 200µl samples of cell free extracts were loaded onto the column and the column run in 0.1M Tris/HCl pH 8.0, 1mM EDTA and 20% (v/v) glycerol at a flow rate of 0.3ml/min. 0.2ml fractions were collected on ice using a Frac 100 fraction collector (Pharmacia LKB) and assayed for CS (Method 3.3) using 20% (v/v) glycerol in the assay mixture. A calibration curve was constructed using a number of proteins of known molecular weights; the Kd values of the proteins were calculated using the equation

$$Kd = \frac{Ve-Vx}{Vy-Vx}$$

Where Ve=elution volume of the protein, Vx=exclusion volume of Blue Dextran 2000 (M_r 3×10^6) and Vy=elution volume of dnp-lysine (M_r 367). The Kd values of the CSs were also calculated and compared with the calibration curve for an estimation of molecular weight.

3.9 Gram Staining

Gram staining was performed as described by Gillies and Dodds (1973). A loopful of bacteria from an overnight culture was spread on a microscope slide. The slide was dried in air and the material fixed by passing the slide three times through the flame of a bunsen burner. The slide was then flooded with Methyl Violet (0.5% w/v) and left for 5 min after which it was rinsed with Gram's Iodine (0.3% w/v iodine, 0.6% w/v potassium iodide). After a further 2 min, the slide was drained and the cells decolourised by exposure to acetone for 5 s before rinsing in water. Neutral red (1% w/v neutral red, 1% v/v HAc) was applied as a counterstain, and after 30 s the slide was rinsed with water and blotted dry.

3.10 Extraction of Nucleic Acids with Phenol:Chloroform

Phenol was removed from storage at -20 °C, warmed to room temperature and then melted at 68 °C. The upper aqueous layer was removed and an equal volume of chloroform:isoamyl alcohol (24:1) was added. To the DNA sample was added an equal volume of this phenol:chloroform mixture and the resulting mixture shaken until an emulsion formed. The mixture was then centrifuged at 12 000 g for 2 min in a microfuge or at 2 000 g for 15 min if a large volume was being extracted. The upper aquous phase containing the DNA was removed taking care not to disturb the protein interface.

3.11 Ethanol Precipitation of Nucleic acids

The sample volume was estimated and one tenth of the volume of 3M NaOAc pH 5.5 was added. Two volumes of ice-cold absolute ethanol was added and, unless otherwise stated, the sample placed at -20 °C until a precipitate had formed. Usually 30 min was sufficient. The DNA was collected by centrifugation at 12 000 g for 10 min in a microfuge or 10 000 g, 4 °C for 30 min if precipitation was being carried out on a larger volume. The pellet was then carefully washed with 70% (v/v) ethanol and dried under vacuum.

3.12 Estimation of DNA quantity by Spectrophotometry

The amount of DNA in a sample was measured by spectrophotometry at 260nm. An absorbance of 1 at 260nm corresponds approximately to 50μg of double stranded DNA and 37μg of single stranded DNA.

3.13 Agarose Gel Electrophoresis

Horizontal submarine gel electrophoresis was used for the analysis of DNA. Unless otherwise stated, 1% (w/v) agarose in TBE was heated until melted and then allowed to cool to about 60 °C. Ethidium bromide was then added to give a final concentration of 0.5μg ethidium bromide/ml. A perspex mould with two solid perspex sides was prepared by forming the remaining two sides with masking tape and placing a comb about 1cm from one end of the mould, making sure that it did not touch the bottom of the mould. The cooled agarose was poured into the mould and allowed to set at room temperature or at 4 °C if a low melting temperature gel was being used. When set, the comb was carefully removed and the gel placed in the main apparatus with the wells towards the cathode. The gel was then covered in running buffer, TBE containing 0.5µg ethidium bromide/ml, unless otherwise stated. The samples were loaded in 15% (w/v) Ficoll, 0.25% (w/v) xylene cyanol, 0.25% (w/v) Bromophenol Blue and electrophoresed at 50V, unless otherwise stated, until the leading dye was at least three quarters of the way down the gel. The DNA was visualised using a transilluminator.

3.14 Isolation of Chromosomal DNA

A 100ml culture of *E.coli* was grown for 16 h and the cells harvested. The supernatant was discarded and the cells resuspended in 40ml TE and centrifuged as before. Again the supernatant was discarded and this time the pellet was resuspended in 3.22ml of 50mM Tris/HCl pH 8.0, 25% (w/v) sucrose. 0.64ml of lysozyme (20mg/ml) was added and the cells swirled gently on ice for 5 min. Then 0.5ml of 20% (w/v) SDS and 0.64ml of 0.5M EDTA pH 8.0 were added and the cells incubated on ice for a further 5 min. 2.5mg of proteinase K were added to the lysed cells which were then incubated at 45 °C for 30 min. The digested protein in the lysate was then removed by phenol extraction (Method 3.10). The aqueous layer was removed and the phenol:chloroform layer re-extracted with 2.5ml of TE. The aqueous layer was once again removed and pooled with that from the previous extraction. The nucleic acids in the aqueous sample were collected by ethanol precipitation (Method 3.11) and dried under vacuum. The pellet was resuspended in 5ml of TE and the nucleic acid was precipitated again with ethanol. The pellet was then washed with 70% (v/v) ethanol and dried under vacuum.

3. 15 Removal of RNA

The dried pellet of isolated DNA from Method 3.14 was resuspended in 4.5ml of water. 0.45ml of 20x SSC and 0.25ml of RNAase A (10mg/ml) (2. 4.3) were added and the mixture incubated at 37 °C for 20 min. The digested nucleic acids were then removed by phenol extraction (Method 3.10). The aqueous layer containing the DNA was removed and the DNA collected by ethanol precipitation (Method 3.11). The precipitate was resuspended in 2ml of water by swirling on ice. 100 μl of 4M NaCl was added and the DNA precipitated and collected as before. The pellet was resuspended in 2 ml of water and ethanol precipitated for a third time. The purified DNA was washed in 70% (v/v) ethanol, dried under vacuum and finally resuspended in 2ml of TE and stored at -20 °C. The quantity of DNA was estimated spectrophotometrically (Method 3.12).

3.16 Restriction Endonuclease Digestion

Purified DNA was mixed with the appropriate buffer (diluted 10 fold to its working concentration) and restriction endonuclease (1 unit/μg DNA) in an Eppendorf tube, ensuring that the enzyme made up no more than 10% of the final volume. The mixture was thoroughly mixed and incubated at 37 °C for 1 h. When double digests were being carried out, the sample buffer was then adjusted for the second enzyme, the enzyme added (1 unit/μg DNA) and the DNA incubated for a further hour at 37 °C. The samples were then analysed by agarose gel electrophoresis (Method 3.13).

3.17 Southern Blotting (Southern, 1975)

After running overnight at 50mA the agarose gel was carefully transferred to a large container and incubated in 0.4M NaOH, 0.6M NaCl, with gentle shaking, for 30 min to denature the DNA. This was replaced with neutralising solution; 0.5M Tris/HCl pH 7.5, 1.5M NaCl, and the gel agitated gently for a further 30 min with one change of neutralising solution. Meanwhile, a piece of NEN GeneScreen plus membrane (Dupont, U.K.) was cut to the same size as the gel. This was then soaked in water for 15 min followed by 10 min soaking in 10x SSC. The apparatus was set up as shown in Fig. 3.1. Care was taken not to trap air bubbles between each layer. Any gel and wicks visible were covered in cling film to prevent short circuiting and evaporation of the buffer. The tray was half -filled with 10x SSC and the transfer allowed to proceed overnight. The next day the paper towels and filter papers were carefully removed from the membrane, which in turn was removed and soaked in 0.4M NaOH for 60 s. The NaOH was replaced with Tris/HCl pH 7.5 and the filter soaked in this for 10 min with one change of buffer. The membrane was removed and air-dried at room temperature between Whatman 3MM for 15 min and placed at 37 °C for 30 min. Finally the membrane was sealed in a plastic bag and kept at -20 °C until required.

3.18 DNA labelling

DNA was labelled using a Multiprime kit (Amersham) based on the random sequence hexanucleotide priming method (Feinberg & Vogelstein, 1983) using Klenow. 1μg of plasmid DNA was cut with the appropriate restriction endonucleases and fractionated on a 1% (w/v) low gelling temperature agarose (Sigma) gel (Method 3.13) using 20mM Tris/HCl pH 8.0, 20mM NaAc, 2mM EDTA, 0.5μg ethidium bromide/ml as the running buffer. The band of interest was cut out of the gel with a minimum amount of agarose and transferred to a pre-weighed Eppendorf tube. The tube was

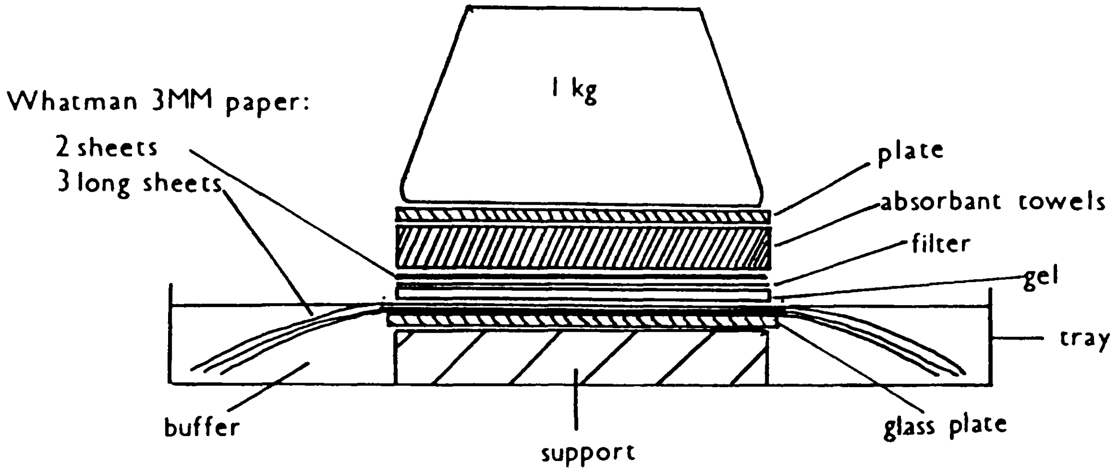


Fig. 3.1 Southern Blotting

then re-weighed and 3ml water/g gel was added. The tube was then placed in a boiling water bath for 7 min to melt the gel. The sample was then divided into aliquots to give at least 25ng DNA per tube and stored at -20 °C until required. Prior to labelling the aliquot was re-boiled for 1 min to denature the DNA and then placed at 37 °C for 10 min. The DNA was then added to the standard Multiprime reaction and labelled for 6 h using [α-^{32}P]dATP. Following the reaction, the sample was passed through a spun Sepharose CL6B (Pharmacia LKB) (Method 3.22) column to remove unincorporated [α-^{32}P]dATP.

3.19 Measurement of Radioactive Incorporation

1µl of labelled DNA was spotted onto the centre of each of two 2.3 cm discs of Whatman DE81 ion exchange paper. One disc was washed three times, for 5 min each wash, in 5% (w/v) Na_2HPO_4, once (1 min) in water and once (1 min) in 95% (v/v) ethanol. Both discs were then dried and counted in a scintillation counter in Optiphase "Safe" scintillant (Pharmacia LKB).

3.20 Hybridisation

50µl of denatured salmon sperm DNA (2.4.4) was added to 25ml of prehybridisation solution (6x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS). The Southern blot (Method 3.17) was transferred to this solution and pre-hybridised, with shaking, at 65 °C for 1 h. The denatured labelled probe (Method 3.18) was added to the pre-hybridisation mix and the blot hybridised at 65 °C overnight. The filter was then washed at 65 °C in 50 ml of 2x SSC for 15 min and then in 50ml of 2x SSC, 0.1% (w/v) SDS at 65 °C for 30 min. The blot was then wrapped in Saran Wrap and autoradiographed (Method 3.21).

3.21 Autoradiography

Fuji RX X-ray film was used for all autoradiography. When filters containing ^{32}P were autoradiographed the film was preflashed, an intensifying screen was used and the film exposed at -70 °C. When gels containing ^{35}S were autoradiographed no preflashing nor intensifying screen was required and the film was exposed at room temperature.

3. 22 Spun Column Chromatography

Using a hypodermic needle (19 G) a small hole was made in the bottom of a 0.5ml Eppendorf tube. Glass beads (0.5mm) were placed in the narrow part of the tube and Sepharose CL6B (Pharmacia LKB) was added to give a column length of about 0.5cm. The small Eppendorf was then placed in a 1.5ml Eppendorf and centrifuged at 2 000 g for 4 min. The liquid in the bottom Eppendorf was discarded and the small tube replaced in the large tube. Two 50μl aliquots of TE were passed through the column by centrifugation as before, the liquid in the bottom tube being discarded each time. The column was then placed in a new 1.5ml Eppendorf, the sample (<50μl) applied to the column and the column centrifuged at 2 000 g for 2 min.

3.23 Small Scale Plasmid Preparation (Minipreps)

The alkali lysis method of Birnboim and Doly (1979) was used. 10ml cultures in LB containing 100μg ampicillin/ml medium were grown for a minimum of 6 h. The cells were harvested and the supernatant discarded. The cell pellet was resuspended in 100μl of freshly-prepared 25mM Tris/HCl pH 8.0, 50mM glucose, 10mM EDTA. 2mg lysozyme per ml cell suspension was added and the cells incubated on ice for 10 min. 200µl of freshly prepared 0.2M NaOH, 1% (w/v) SDS were added and after mixing gently the cells were lysed on ice for 10 min. The cell contents were then neutralised by mixing with 150µl of 3M KOAc/5M HAc pH 4.8 and incubated on ice for 10 min. After centrifuging at 15 000 g for 2 min at 4 °C the supernatant was transferred to a new Eppendorf tube and ethanol precipitated (Method 3. 11) on ice for 10 min. After washing and drying, the DNA was resuspended in 20µl of TE.

3.25 Large Scale Plasmid Preparations (Maxipreps)

500ml cultures in LB containing 100µg ampicillin/ml medium were grown overnight. The cells were harvested and then resuspended in 8ml 25mM Tris/HCl pH 8.0, 50mM glucose, 10mM EDTA. 0.5mg lysozyme was added per ml cell suspension and the cells were then disrupted by incubation on ice for 15 min followed by the addition of 16ml of freshly-prepared 0.2M NaOH, 1% (w/v) SDS and incubation on ice for 30 min. 12ml 3M KOAc/5M HAc pH 4.8 were added and the cells incubated on ice for a further 10 min. Chromosomal DNA was removed by centrifugation at 3 000 g 4 °C for 15 min. The supernatant was transferred to clean tubes and the DNA precipitated at room temperature by the addition of 0.6 times the volume of isopropanol. The DNA was recovered by centrifugation at 10 000 g 4 °C for 30 min. The DNA pellet was dried under vacuum and further purified by caesium chloride density gradient (Method 3.26).

3.25 Caesium Chloride Density Gradient

The dried DNA sample was resuspended in 9.5ml of TE. 9.5g of caesium chloride (CsCl) were added and when dissolved, 0.3ml of a solution of 10mg ethidium bromide/ml was added. The solution was transferred to a heat-sealable tube (Beckman) and filled with a 1g CsCl/ml solution. The tube was sealed and centrifuged for 16 h at 200 000 g in a Ti70.1 rotor (Beckman). After carefully removing the tube from the centrifuge, the lower of the two bands was removed using a hypodermic syringe and needle (18G). The ethidium bromide was removed by shaking the sample with an equal volume of CsCl-saturated 2-propanol. The upper phase was removed and the lower phase was re-extracted with CsCl-saturated 2-propanol until no trace of pink remained and then once more. An equal volume of water was added to the DNA solution and the DNA precipitated with ethanol at room temperature (Method 3.11). After drying, the pellet was resuspended in 1ml TE and the quantity of DNA estimated by spectrophotometry (Method 3.12).

3.26 Oligonucleotide Preparation

Oligonucleotides were prepared using an automated synthesiser (Applied Biosystems) based on the phosphoramidite method (Matteucci & Caruthers, 1980; Beaucage & Caruthers, 1981; Matteucci & Caruthers, 1981). Upon completion of the synthesis, the column was removed from the synthesiser and to one end was fixed a 5ml syringe and to the other was fixed a 1ml syringe containing 1ml of concentrated ammonia. 0.3ml aliquots of ammonia were drawn into the column every 20 min until all the ammonia had passed through. The solution was then placed in a screw capped tube and incubated at 55 °C overnight. The oligonucleotide was dried under vacuum and then resuspended in 1ml water and ethanol precipitated (Method 3.11) at -70 °C. After drying under vacuum, the oligonucleotide was resuspended in 1ml of water and the quantity estimated by spectrophotometry (Method 3.12).

3.27 Amplification of Chromosomal DNA (Saiki *et al.*,1985)

Amplification was carried out in a final volume of 100µl containing 100ng purified chromosomal DNA or 10ng purified plasmid DNA, 10mM Tris/HCl pH 8.3, 50mM KCl, 3mM MgCl, 200µl each dNTP, 0.1mM oligonucleotides and 2.5 units *Thermus aquaticus* (*Taq*) DNA polymerase (Chien *et al.*, 1976) (Perkin-Elmer Cetus). The mixture was overlayed with 100µl of mineral oil (Sigma) and amplification carried out for 30 cycles in the PHC 2 (Techne). The DNA was denatured at 95 °C for 1 min, the oligonucleotides annealed for 1 min at 45 °C and the DNA extended at 72 °C for 1.5 min. 10µl of the reaction mix was analysed by agarose gel electrophoresis (Method 3.13).

3.28 Filling in Recessed 3′ Ends

To a 20µl sample of amplified DNA was added 2µl of 10x NTB (0.5M Tris/HCl pH 7.5, 0.1M $MgSO_4$, 1mM EDTA, 500ng BSA/ml), 1µl of dNTP mix containing each dNTP at 2mM and 1 unit of Klenow. After mixing,the solution was incubated at room temperature for 30 min and the reaction stopped by the addition of 1µl of 0.5M EDTA pH 8.0.

3.29 Sticky-End Ligation

A total of 50ng of vector and insert DNA, in the molar ratio 0.5:1, were ligated together. The reaction mix (20µl) containing 50mM Tris/HCl pH 7.5, 10mM $MgCl_2$, 1mM DTT, 1mM ATP and 1 unit of ligase was incubated at room temperature for 4 h. 2µl of the ligation reaction were then diluted ten times with TE and this was used to transform competent bacterial cells (Method 3.30).

3.30 Competent Cells

A single colony of the *E.coli* bacterium TG1 was removed from a minimal medium plate and used to inoculate 10ml LB. This was grown overnight and 0.5ml of this culture was used to inoculate 50ml of LB which was then incubated at 37 °C. When the cells had reached early log phase of growth they were removed from the incubator and placed on ice for 10 min. The cells were then harvested and gently resuspended in 25ml of ice-cold 50mM $CaCl_2$. The cells were then incubated on ice for 30 min and centrifuged as before. The cells were then gently resuspended in 3ml of ice cold 50mM $CaCl_2$, and kept on ice until required.

3.31 Transformation of Competent Cells

0.2ml of competent cells were dispensed into a chilled Eppendorf tube on ice. 20μl of diluted ligation mix (Method 3.29) was added to the cells and mixed by inversion. The mixture was then incubated on ice for 30 min. The cells were then heat shocked by placing at 42 °C for 90 s and returned to ice for 5 min. 1ml of LB was added and the cells incubated at 37 °C without shaking for 30 min to allow plasmid establishment. 200μl of transformed cells together with 50μl of 100mM IPTG and 10μl of X-Gal (2% w/v) were spread onto an LB plate containing 100μg ampicillin/ml and incubated at 37 °C. The degree of competence of the cells was measured by transformation with 4ng of pUC19 supercoiled plasmid and plating out 250μl of the transformed cells. Each colony obtained then represented a competence of 1000 transformants/μg DNA.

3.32 Production of Single Stranded Sequencing Templates from Double Stranded DNA

Immediately prior to sequencing, 3-5μg of plasmid DNA in a total volume of 20μl were denatured by reaction with 5μl of 1M NaOH, 1mM EDTA pH 8.0 for 1 min at room temperature. The alkali was removed by spun column chromatography (Method 3.22) using Sepharose CL6B (Pharmacia LKB).

3.33 Sequencing

Sequencing reactions were performed using Sequenase version 2.0 kit (United States Biochemical Corp.) (Tabor & Richardson, 1987) which is based on the chain-termination DNA sequencing method (Sanger *et al.*, 1977). 10 μl [α-^{35}S]dATP were used in each labelling reaction and the termination reactions were carried out at 45 °C to prevent 'pausing' at sites of exceptional secondary structure.

3.34 Denaturing Gel Electrophoresis

An 8% (w/v) polyacrylamide mix was made using Sequagel reagents (National Diagnostics) and a 0.4mm-thick gel poured using the sliding plate technique. A sharks tooth comb was inserted upside down into the gel and the gel allowed to polymerise at room temperature. Immediately prior to loading, the sequencing reactions were denatured by heating to 75 °C for 2 min. The comb was placed in the gel the right way up and 5μl of reaction mix were loaded into each well. The gel was run at 1 200V, 30mA for the appropriate amount of time in TBE buffer. The top plate was then levered off and the gel soaked in 10% (v/v) methanol, 10% (v/v) HAc for 20 min. After carefully removing excess liquid from the gel,a piece of Whatman 3MM paper cut to size was carefully laid onto the gel and after complete contact was made, the paper carefully lifted with the gel stuck to it. The gel was then wrapped in cling film and dried under vacuum at 80 °C for 45 min. The cling film was removed and the gel autoradiographed.

3.35 Rapid Lysis Technique

1.5ml of an overnight culture was centrifuged at 12 000g for 1 min. The pellet was immediately resuspended in 150µl of sample buffer (Method 3.36) by vortexing vigorously. The sample was then placed in a boiling water bath for 3 min and the cell debris was then removed by centrifugation at 12 000 g for 1 min.

3.36 SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of SDS was performed using the discontinuous method of Laemmli (1970).

A 10% (w/v) polyacrylamide slab gel was poured and overlaid with water. When set the water was removed and a 3.75% (w/v) polyacrylamide stacking gel poured on top and a comb placed in the stacking gel. 0.375M Tris/HCl pH 8.8 and 0.125M Tris/HCl pH 6.8 were used for the separating and stacking gels respectively. Polymerisation was achieved in both cases by using ammonium persulphate and N,N,N',N-tetramethylethylenediamine (TEMED).

Protein solutions were mixed with loading buffer (0.1M Tris/HCl pH 8.9, 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol and 0.001% (w/v) Bromophenol blue) and boiled for 5 min prior to loading. Molecular weight markers (Pharmacia LKB) were made up according to the manufacturers instructions and also boiled prior to loading.

The gel was run overnight at 50V using 0.025M Tris pH 8.3, 0.192M glycine, 1% (w/v) SDS as running buffer.

3.37 Protamine Sulphate Precipitation

Nucleic acids were removed from cell free extracts by treatment with protamine sulphate. A 1% (w/v) protamine sulphate solution was added dropwise, with stirring, to the cell free extract (1mg protamine sulphate/10 mg protein) in ice. After addition of the protamine sulphate the extract was stirred in ice for a further 30 min. The precipitated nucleic acids were then collected by centrifugation at 20 000 g, 4 °C for 30 min.

3.38 Staining and Destaining Gels

SDS-polyacrylamide gels were stained by shaking for 2 h in 0.1% (w/v) Coomassie Brilliant Blue in 30% (v/v) methanol, 10% (v/v) HAc. Destaining was carried out in several changes of 30% (v/v) methanol, 10% (v/v) HAc.

3.39 Production of Polyclonal Antibody to Citrate Synthase

300µg of purified CS in 20mM Tris/HCl pH 8.0 were mixed 1:1 with Freunds complete adjuvant in glass syringes. A 10 ml test bleed was taken from a male rabbit and the CS injected at two intramuscular sites. Two boosts of 200µg of CS mixed with Freunds incomplete adjuvant were given at days 17 and 28, again at two intramuscular sites. On day 35 a second 10ml bleed was taken.

3.40 Removal of Serum from Blood Samples

After removal of the sample the blood was left at room temperature for 1 h and then at 4 °C overnight to clot. The sample was centrifuged at 2 500 g for 15 min at 4 °C. The serum was removed and aliquots kept at -20 °C until required.

3.41 Immuno Dot Blots

A piece of nitrocellulose (Schleicher and Schüll) was cut to fit in a filtration manifold (Schleicher and Schüll) and pre-wetted together with a filter pad by soaking in water for 5 min. The filter and nitrocellulose, marked for orientation, were then placed on the manifold and the manifold clamped tightly. A 30µl sample of antigen, diluted with 70µl PBS, was applied to each well and allowed to remain on the nitrocellulose without suction for 30 min. Slight suction was then applied for 30 s. The nitrocellulose was removed and air dried before staining and detection.

3.42 Western Blotting

A piece of nitrocellulose (Schleicher and Schüll) and two pieces of Whatman 3MM paper were cut to exactly the size of the polyacrylamide gel. These were soaked in transfer buffer, 48mM Tris, 39mM glycine, 20% (v/v) methanol (Analar grade), 0.375% (w/v) SDS for 30 min. One of the sheets of Whatman 3MM was placed on the anode plate of a semi dry electrophoretic transfer cell (BioRad Laboratories). The nitrocellulose was placed on top of this, followed by the gel and finally the second piece of Whatman 3MM. Care was taken to ensure that no air bubbles were trapped between the layers. The cathode was clamped in place and transfer was carried out at 350mA for 45 min.

3. 43 Staining and Detection of Immunoblots

After immunoblotting, the nitrocellulose was washed twice in PBS. Non-specific sites were blocked by incubation, with shaking, for 2 h in 3% (w/v) BSA in PBS at room temperature. The blot was then incubated overnight with serum diluted 1:1000 with 3% (w/v) BSA in PBS. After washing the blot four times with PBS, anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase (Sigma) at a 1:5000 dilution in 3% (w/v) BSA in PBS, was added. The blot was incubated for a further 2 h at room temperature after which time the blot was washed four times in PBS.

Immediately prior to use 10mg 3-3′-diaminobenzidine was dissolved in 15ml of 50mM Tris/HCl pH 7.6. 12µl of fresh 30% (v/v) H_2O_2 was added and the solution added to the blot. After the colour had developed, the reaction was stopped by rinsing in PBS.

3.44 Transfer of Proteins for N-Terminal Sequencing

Following electrophoresis by SDS-PAGE (Method 3.37), the gel was equilibrated for 15 min in transfer buffer, 50mM Tris, 50mM glycine, 20% (v/v) methanol (Analar grade). At the same time a piece of Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) cut to size and two pieces of Whatman 3MM paper also cut to size were soaked in transfer buffer. The equilibrated gel was placed in contact with the membrane and electroblotted for 15 h at 200mA at 4 °C in transfer buffer.

4. PRODUCTION AND SELECTION OF CITRATE SYNTHASE REVERTANTS

4.1 Introduction

The citric acid cycle is at the centre of metabolism in that it not only acts as the final pathway for the oxidation of major foodstuffs, but also provides the precursors for amino acid and porphyrin biosynthesis. Citrate synthase (CS), has been proposed as the key regulating enzyme in this major metabolic pathway and is itself regulated by several factors. This diversity in the regulatory properties of CS, probably reflects the different physiological demands on the citric acid cycle.

In situations when the cycle is used solely for the production of the biosynthetic precursors 2-oxoglutarate and succinyl-CoA, it is these that control the level of CS (Weitzman & Dunmore, 1969; Taylor, 1973; Lucas & Weitzman, 1975). However, when the cycle is used exclusively for the production of energy, such as in strict aerobes, AMP serves as the regulator of CS (Weitzman & Jones, 1968). In the majority of cases, however, the citric acid cycle performs both an energy producing and biosynthetic role and, therefore, does not fall into either of the categories above. In these situations the most notable difference in the regulation of CS is the division between those sensitive to NADH and those that are apparently unaffected by this regulator. This regulation appears to be at the quaternary protein structure. That is, that the 'large' type enzymes from Gram-negative organisms are NADH sensitive whereas the 'small' type enzymes from eukaryotes and Gram-positive bacteria are NADH-insensitive. The question of what features of the quaternary structure contribute to this diversity need to be answered.

Direct comparisons between the protein sequences of the 'large' and 'small' type enzymes are of limited value in that evolution may well have introduced neutral changes in the protein sequence as well as those that alter the enzyme's structure and regulation. Mutations producing minor genetic changes but which result in major structural and regulatory changes of the enzyme, have provided an invaluable tool with which to investigate the relationship between CS structure and regulation. Danson and colleagues (Danson *et al.*, 1979) have already produced a mutant of *E.coli* that produces a 'small' rather than 'large' CS. This mutant enzyme differed from the wild type enzyme not just at the molecular level but also displayed regulatory characteristics that were consistent with a 'small' type enzyme. The aim of the work in this chapter was to recreate this mutant in order to investigate it further, at both the protein and molecular biological level.

4.2 Mutation Strategy

The strategy followed that previously used by Harford (1977) to create a mutant CS producing strain of *E.coli.* The parent strain of *E.coli* used for the mutation was K114. K114 lacks CS activity and was derived by mutation of *E.coli* K12.

K114 was mutated by exposure to the alkylating agent ethyl methanesulphanate (EMS) for 60 min (Method 3.7). Revertants that had regained CS activity were then selected for, by virtue of their glutamate auxotrophy, by growth on glucose (10mM) minus glutamate plates. This was based on the fact that if CS activity had been restored, 2-oxoglutarate would be produced by the citric acid cycle and could therefore be metabolised to glutamate, thus relieving the requirement for supplementation with this amino acid.

RESULTS

4.3 Production and Characterisation of a Revertant of K114

4.3.1 Reversion of K114

41 glutamate auxotrophs were obtained after EMS exposure and these were individually screened for CS activity. In order to investigate if any of these revertants were potentially of the 'small' type, the effect of 2-oxoglutarate on CS activity was also measured, as 'small' CSs are not regulated by 2-oxoglutarate (Weitzman & Dunmore, 1969). 2-oxoglutarate at a concentration of 5mM was used in this investigation as this concentration has previously been shown to produce virtual total inhibition of the wild type enzyme (Danson *et al.*, 1979). 38 of these revertants showed CS activity and all these were inhibited by 2-oxoglutarate in the range 56-100% inhibition. As glucose has been shown to suppress the levels of CS to virtually zero in mutant strains of *E.coli* (Gray *et al.*, 1966; Else, 1986) it was decided to grow the three colonies not displaying CS activity, in minimal medium with succinate (10mM) as the energy source. CS activity was now detected in these three revertants and was not inhibited by 2-oxoglutarate. K114 failed to grow in minimal medium with succinate as the energy source indicating that these were true revertants.

4.3.2 Molecular Weight Estimation

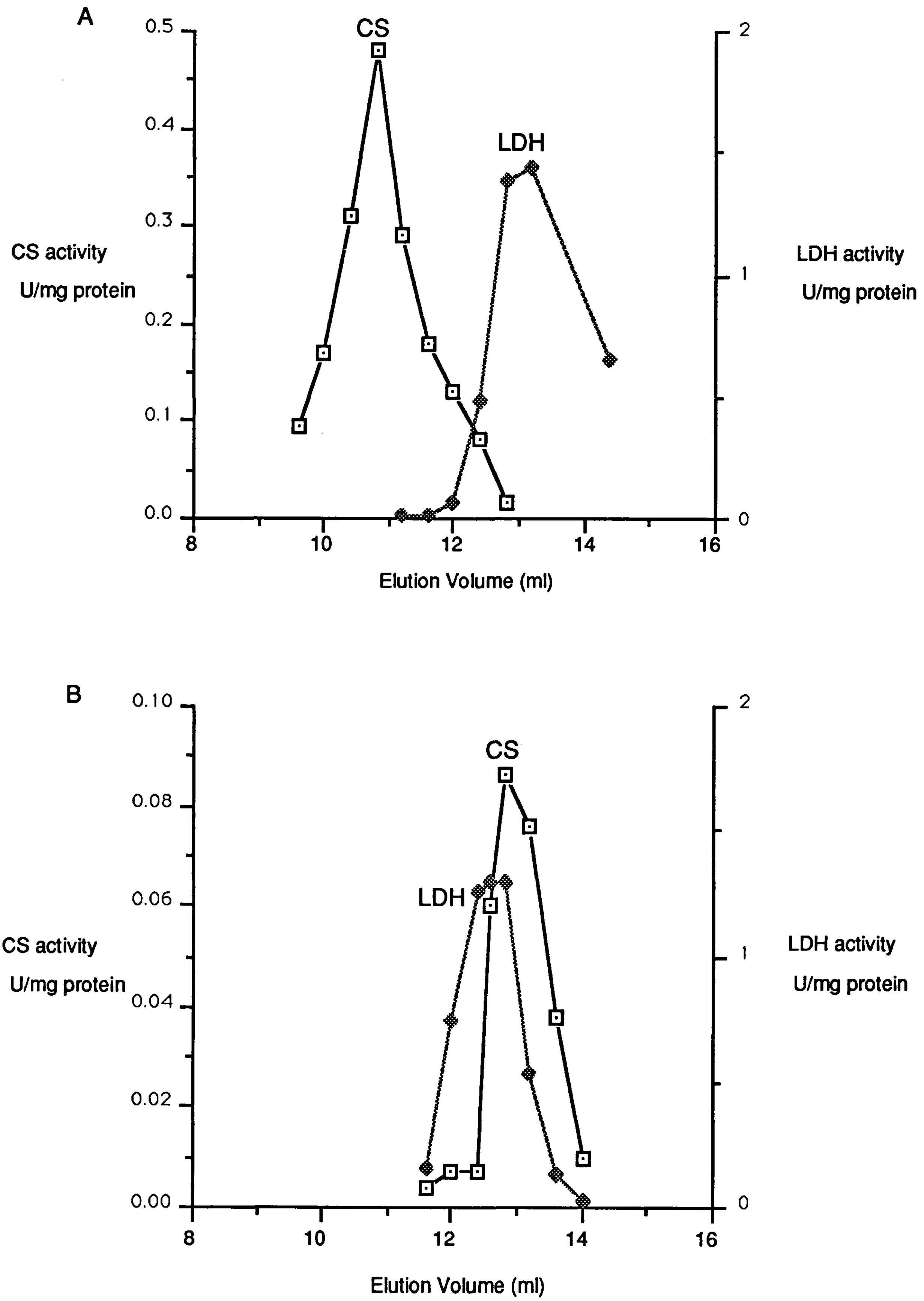
Cell extracts of the three revertants whose CSs were not inhibited by 2-oxoglutarate were then applied to a Pharmacia LKB Superose 12 gel filtration column (FPLC) for molecular weight estimation (Method 3.8). The cell free extracts were supplemented with 50 μl of LDH (rabbit muscle) to serve as a marker. LDH has a M_r ~140 000 and, therefore, if the CS under investigation was of the 'large' type (M_r~250 000) it would elute prior to LDH from the column and if it was of the 'small' type (M_r~100 000) it would elute with or after LDH. The fractions were, therefore, assayed for both CS (Method 3.3) and LDH (Method 3.4) activity. One of the three eluted prior to LDH suggesting that it was of the 'large' type (Fig. 4.1A) whilst the other two eluted just after LDH indicating that they were of the 'small' type (Fig. 4.1B). The Kd values of these CSs were calculated to be 0.28 and 0.3 which when compared with the standard curve (Fig. 4.2), gave estimated molecular weights of 118 000 kDa and 110 000 kDa respectively. One of these revertants (M_r~118 000) was far more stable than the other and was, therefore, a better candidate for further investigation. This revertant, now referred to as K114r4 was further examined to establish whether it had characteristics comparable with those reported for previous 'small' *E.coli* CS revertants.

4.3.3 Kinetic Properties of K114r4

The specific activity of CS from cell free extracts of K114r4 was shown to be 0.04 U/mg protein compared with 0.12 U/mg protein for CS from cell free extracts of HB101, a wild type *E.coli.* HB101 was used as the parent strain of K114 was unavailable.

CS activity from K114r4 showed hyperbolic dependences of rate on both acetyl-CoA and OAA concentrations (Fig. 4.3). The data was analysed by a computer program based on the direct linear plot (Eisenthal & Cornish-Bowden, 1974) and are presented as half-reciprocal plots (Fig. 4.4). These plots give straight lines indicating that the enzyme obeys the Michaelis-Menten equation. Analysis of the data gave apparent K_m values of 10.9 (±4.15) μM and 7.0 (±1.9) μM for acetyl-CoA and OAA respectively. This was in contrast to CS from HB101 which displayed sigmoidal dependences of

Fig. 4.1. Elution Profiles of (A) 'large' CS and (B) 'small' CS from a Superose 12 gel filtration column. LDH was used as a marker as this has a molecular weight similar to 'small' CSs.


A
CS
LDH
CS activity
U/mg protein
LDH activity
U/mg protein
0.5
0.4
0.3
0.2
0.1
0.0
2
1
0
8
10
12
14
16
Elution Volume (ml)
B
CS
LDH
CS activity
U/mg protein
LDH activity
U/mg protein
0.10
0.08
0.06
0.04
0.02
0.00
2
1
0
8
10
12
14
16
Elution Volume (ml)

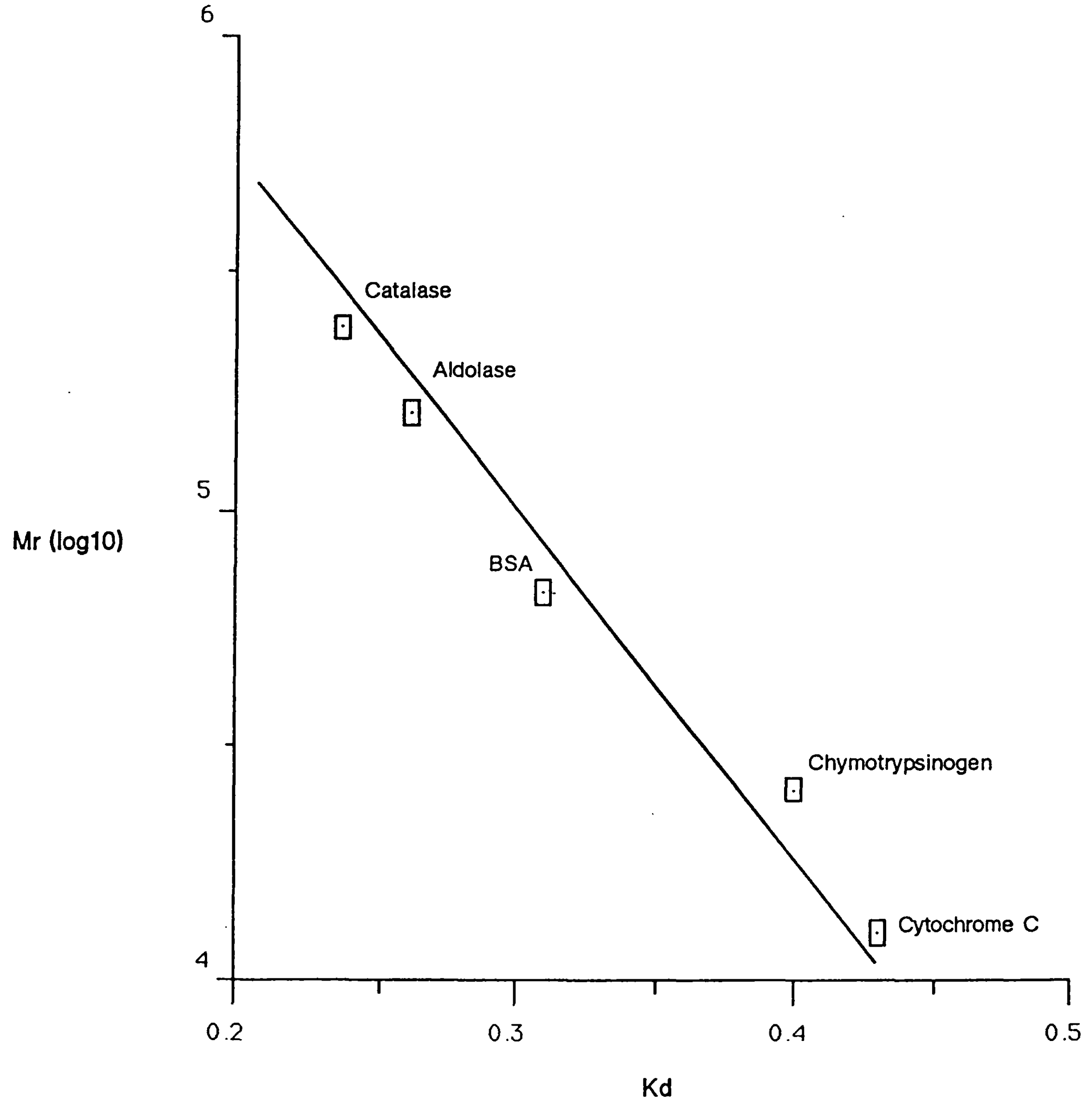
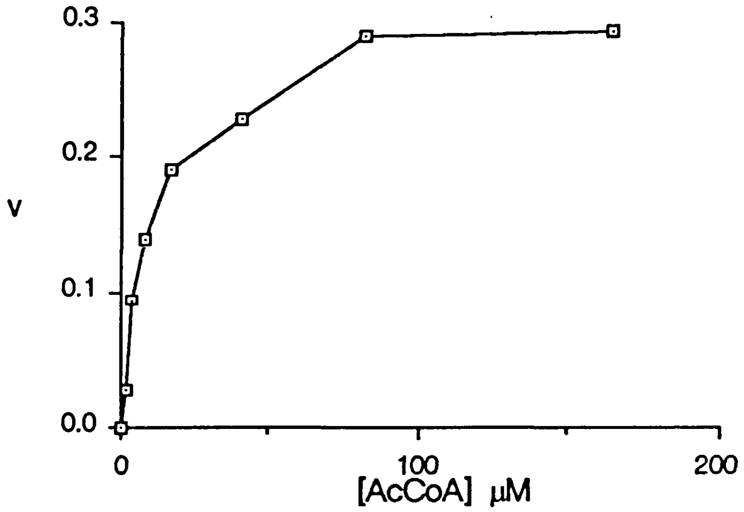


Fig. 4.2. Molecular Weight Calibration Curve



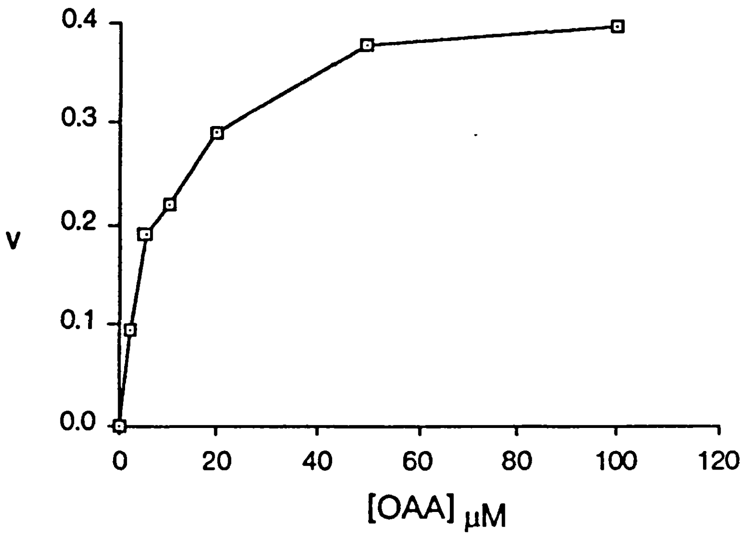
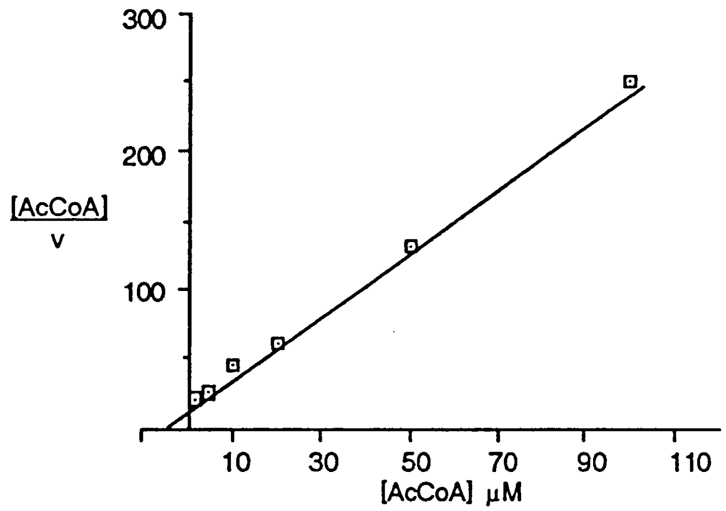


Fig. 4.3. Relationship of Reaction Rate (v) versus [S] for Citrate Synthase from *E.coli* K114r4



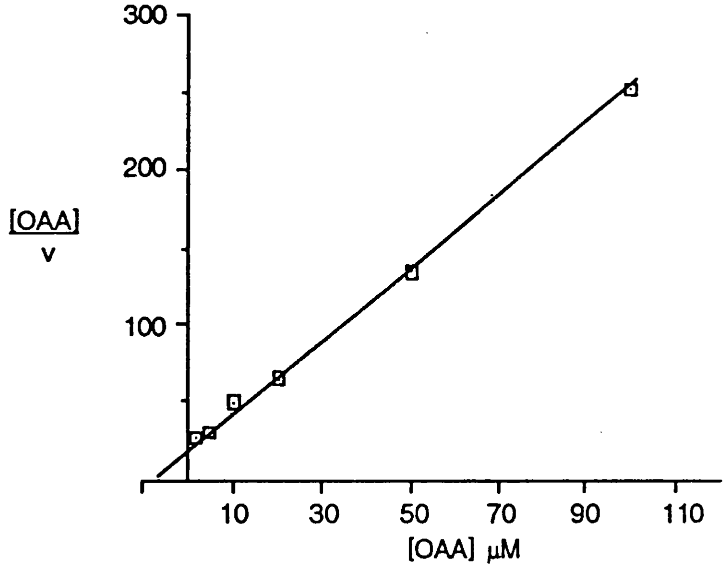
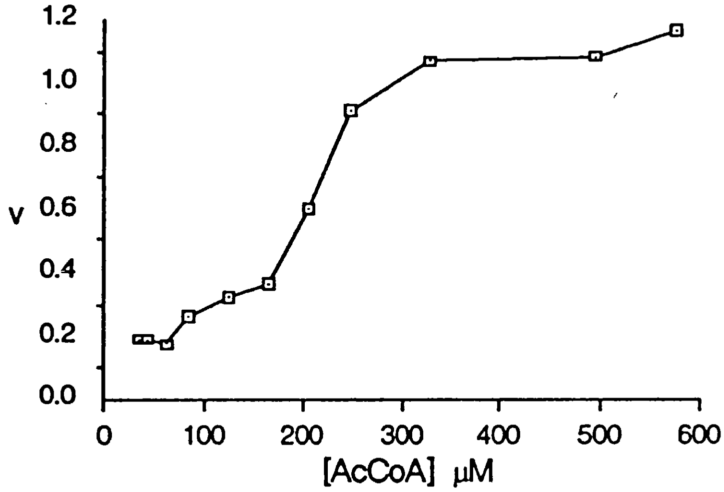


Fig. 4.4. Hanes Woolf Plot: [S]/v versus [S] for

Citrate synthase from *E.coli* K114r4



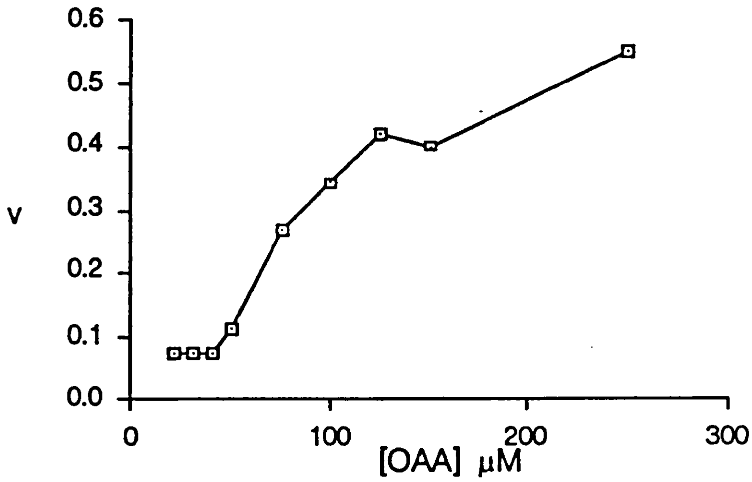


Fig. 4.5. Relationship of Reaction Rate (v) Versus [S] for

Citrate Synthse from *E.coli* HB101

rate on both acetyl-CoA and OAA concentrations (Fig. 4.5), though the sigmoidicity was more pronounced for acetyl-CoA. In addition, $S_{0.5}$ values obtained from best fits of the data using a computer program based on the Hill equation were much higher than those obtained for the mutant enzyme, 197μM and 94μM for acetyl-CoA and OAA respectively.

4.3.4 Regulatory Properties of K114r4

2-oxoglutarate has been shown to be inhibitor of *E.coli* CS (Weitzman & Dunmore, 1969). At a concentration of 2mM, 2-oxoglutarate produced 90% inhibition of the activity of the CS from cell free extracts of HB101, whereas no inhibition of the enzyme was produced from cell free extracts of K114r4. Inhibition (30%) of CS activity from K114r4 was seen only when a concentration of 2-oxoglutarate as high as 10mM was used.

In order to investigate the effect of NADH on the revertant CS enzyme, contaminating malate dehydrogenase (MDH) had to be removed first. MDH cayalses the reaction

$$\text{OAA} + \text{NADH} \leftrightarrow \text{Malate} + \text{NAD}^+$$

Therefore, if present when the effect of NADH on CS was being investigated it would remove all the NADH and OAA added. Gel filtration did not remove contaminating MDH as it has a similar molecular weight, M_r~72 000 kDa, as the 'small' CS. Affinity chromatography using Cibacron Blue Sepharose (Amicon) was tried as a method for removing the contaminating MDH as Cibacron Blue is well known to bind dehydrogenases (Thompson & Stellwagen, 1976). Unfortunately, this proved unsuccessful as the CS was not recovered under these conditions. This was consistent with earlier reported findings (Robinson, 1984). It was, therefore, decided to try to determine the effect of NADH on the revertant CS in the presence of MDH by using a large excess of both NADH (1mM) and OAA (1mM). As these substrates were in excess there would still be sufficient present during the time required to assay CS and examine the effect of NADH on the enzymatic activity. The amount of NADH present at any one time was calculated from the reaction curve of MDH using 6 220 $l.mol.^{-1}cm^{-1}$ as the molar extinction coefficient of NADH at pH 8.0. At a concentration of NADH ≥0.2mM, CS activity from HB101 was inhibited by 70% whereas no inhibition of CS activity from K114r4 was displayed at this concentration of NADH.

4.3.5 Sulphydryl Modification

Chemical modification studies using DTNB have shown that thiol groups are essential for the catalytic activity of wild type *E.coli* CS (Danson & Weitzman, 1973; Danson & Weitzman, 1977; Duckworth *et al.*, 1987). Moreover, the mutant enzyme produced by Danson *et al.* (1979) was shown to be even more sensitive to thiol modification, being more or less totally inactivated after only 1 min exposure to DTNB ([0.1mM]). OAA ([0.2mM]) was shown to provide complete protection of the mutant enzyme against inactivation by DTNB. The reaction of the mutant enzyme from K114r4 to DTNB was investigated to determine whether it reacted in a similar fashion to the mutant enzyme produce by Danson *et al.* (1979). The enzyme was mixed with the standard reaction mixture (Method 3.3) ([DTNB] =0.1mM) but without OAA. After 1 min OAA was added and the enzyme activity measured. The mutant enzyme reacted very quickly with the thiol modifier as no detectable activity was observed after this 1 min exposure to DTNB. Under the same conditions, wild type *E.coli* still retained 92% of its activity after 1 min incubation with DTNB. That is 0.24 U/mg protein after incubation with

DTNB compared with 0.262 U/mg protein before incubation. As OAA protects the mutant enzyme from inactivation by DTNB, OAA was added to the reaction mix prior to DTNB when assaying CS from K114r4.

4.4 Identification of K114r4

Because the CS from K114r4 very closely resembled that from a Gram-positive organism, it was necessary to verify that the organism still retained the characteristics of *E.coli*, and the growth characteristics of K114.

4.4.1 Test for *E.coli*

Gram staining and microscopic examination (Method 3.9) of the organism revealed the appearance of Gram-negative rods. The organism also grew as pink colonies on MacConkey's agar, on account of lactose fermentation, characteristic of *E.coli.*

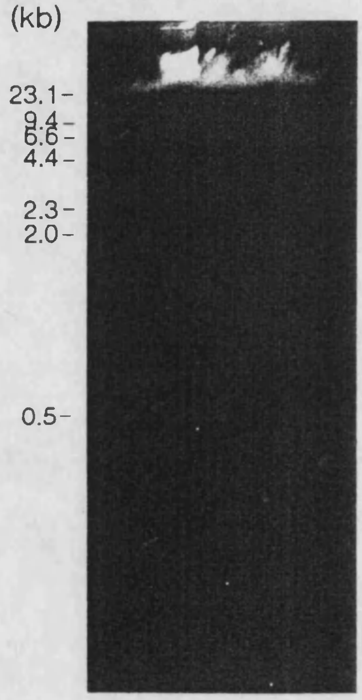
4.4.2.Test for Origination from K114

K114 has an absolute requirement for methionine (20mg/l) and thymine (20mg/l) and K114r4 did not grow in minimal medium without supplementation with both of these substrates.

4.5 Identification of the CS gene

The gene encoding for the wild type CS is known as the *glt*A gene and has been located on a 3.2kb *Hind* III-*EcoR* I fragment (Guest,1981). Chromosomal DNA was first purified (Method 3.14) from the *E.coli* strains K114r4 and HB101. Analysis on an agarose gel (Method 3.13) showed it to be all of high molecular weight without contaminating RNA (Fig. 4.6) indicating that the method produced good quality DNA. Double restriction

Fig. 4.6. Agarose gel (1% w/v) electrophoresis of chromosomal DNA from K114r4. Size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.


(kb)
23.1-
9.4-
6.6-
4.4-
2.3-
2.0-
0.5-

digests (Method 3.16) of these chromosomal DNA preparations were carried out using *Hind* III and *EcoR* I restriction endonucleases. At the same time DNA from the plasmd pDB2, (the *glt*A gene subcloned into pBR322 (Bloxham *et al.*, 1983)), was digested using the same two enzymes to serve as a marker of the *glt*A gene. The digested samples were then run on an agarose gel overnight at 50mA. Restriction digests of the DNA from K114r4 and HB101 with *Hind* III and *EcoR* I appeared to give a similar pattern to each other (Fig. 4.7A). The overnight gel was transferred to nitrocellulose by Southern blotting (Method 3.17). The blot was probed (Method 3.20) using a whole CS gene probe made from the 3.2 kb *Hind* III-*EcoR* I fragment of pDB2 (Method 3.18) and autoradiographed (Method 3. 21).

From the autoradiograph (Fig. 4.7B) the whole gene probe bound to a fragment on the K114r4 and HB101 digests at a region that matched the cloned 3.2 kb fragment of pDB2 containing the *glt*A gene.

4.6 Discussion

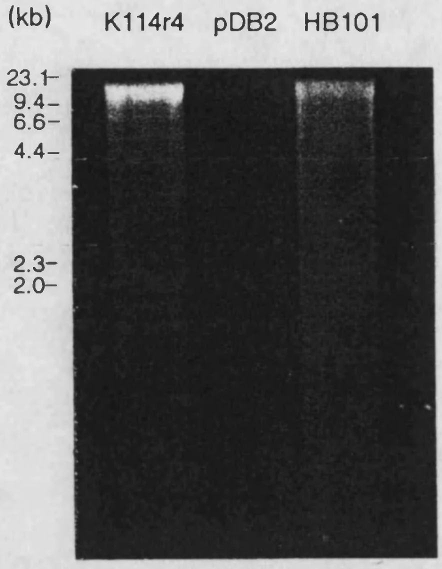
From these results it would appear that isolation of a revertant of *E.coli* that produces CS, from a strain not displaying CS activity, has been successful. Moreover, this revertant exhibits several characteristics that immediately distinguish it from the wild type enzyme.

Firstly the enzyme has been shown to be of the 'small' rather than 'large' type of enzyme as produced by the wild type *E.coli.* The molecular weight estimate obtained is comparable with that of other CSs of Gram-positive bacteria and eukaryotic organisms (Weitzman & Dunmore, 1969).

The fact that the enzyme is not inhibited by 2-oxoglutarate and NADH is also consistent with that found in other 'small' CSs (Weitzman & Dunmore, 1969) and distinguishes it from CS found in wild type *E.coli.*

Fig. 4.7. A Agarose gel (1% w/v) electrophoresis of *Hind* III-*Eco* RI restriction digests of chromosomal DNA prepared from K114r4 and HB101 and plasmid DNA prepared from pDB2 (*glt* A in pBR322). Size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.

Fig. 4.7. B Autoradiograph of DNA samples. Following agarose gel (1% w/v) electrophoresis of *Hind* III-*Eco* RI restriction digests of chromosomal DNA prepared from K114r4 and HB101 and plasmid DNA prepared from pDB2 (*glt* A in pBR322) the DNA was transferred to Genescreen plus membrane by Southern blotting. The membrane was hybridised with a ^{32}P labelled probe made by Multiprime labelling of the *glt* A gene. The size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.


(kb)
K114r4
pDB2
HB101
23.1
9.4
6.6
4.4
2.3
2.0


(kb)
K114r4
pDB2
HB101
6.6
4.4
2.3
2.0

DTNB reacts with free thiols and it has been demonstrated that chemical modification of the wild type CS with this reagent results in a decrease in activity and response to NADH (Danson & Weitzman, 1973). The mutant *E.coli* CS created previously was shown to be extremely sensitive to DTNB (Danson *et al.*, 1979) and the revertant created here was affected in the same way. This could presumably be the result of exposure of essential thiol groups normally buried in the wild type enzyme.

In addition K_m values obtained in this work are similar to those demonstrated in previous work on a mutant CS from *E.coli* (Danson *et al*, 1979) and are significantly different from the wild type enzyme.

From autoradiography results the *glt*A gene is still intact and located on a 3.2 kb *Hind*III-*Eco*R1 fragment and, therefore, it is speculated that the dramatic changes in characteristics of the enzyme have been brought about by minor genetic alterations.

Thus, a mutant has been produced from *E.coli*, presumably by minor genetic changes that exhibits characteristics associated with a 'small' type enzyme and, can, therefore, be investigated further to establish any structure-function relationships.

5. CLONING, SEQUENCING AND EXPRESSION OF THE CITRATE SYNTHASE GENE FROM *E.coli* K114r4, K114 and TG1.

5.1 Introduction

The gene encoding CS in *E.coli* is known as the *glt*A gene and is located at 16.2 min on the *E.coli* linkage map (Ashworth *et al.*, 1965). The *glt*A gene has been located on an approximately 3.2 kb *Hind*III-*EcoR* I fragment (Guest, 1981) which has been cloned and sequenced (Ner *et al.*, 1983) (Fig 5.1).

In the previous chapter it was shown that a mutant of *E.coli* (K114r4) has been created that produces a CS more reminiscent of that produced by eukaryotes and Gram-positive bacteria than by Gram-negative bacteria. The *glt*A gene of the mutant is still located on a 3.2 kb *Hind*III-*EcoR* I fragment suggesting that no major changes, such as deletions, have been made to the *glt*A gene.

In order to identify the nucleotide substitutions responsible for this dramatic change in characteristics it will be necessary to clone and sequence the *glt*A gene encoding the mutant enzyme. In addition, K114r4 is a slow growing organism and the mutant enzyme is very unstable. Thus, a clone containing amplified levels of the mutant gene would facilitate purification of the protein and thus permit a more detailed investigation into the structure-function relationships of the enzyme.

It follows then that the aim was now to clone the *glt*A gene from the mutant K114r4, to sequence the gene and to compare the sequence with the *glt*A gene from the wild type *E.coli.*

5.2 Cloning Strategy

The strategy decided upon was to isolate and amplify the *glt*A gene from chromosomal DNA by automated DNA amplification using primers

```
                                                                                        M  A
5'—AACGGGTAAAGCCAGGTTGATGTGCGAAGGCAAATTTAAGTTCCGGAGTCTTACGGCAATAAGGCGCTAAGGAGACCTTAAATGGC
        230       240       250       260       270       280       290       300       310

 D  T  K  A  K  L  T  L  N  G  D  T  A  V  E  L  D  V  L  K  G  T  L  G  Q  D  V  I  D  I
TGATACAAAAGCAAAACTCACCCTCAACGGGGATACAGCTGTTGAACTGGATGTGCTGAAAGGCACGCTGGGTCAAGATGTTATTGATAT
        320       330       340       350       360       370       380       390       400

 R  T  L  G  S  K  G  V  F  T  F  D  P  G  F  T  S  T  A  S  C  E  S  K  I  T  F  I  D  G
CCGTACTCTCGGTTCAAAAGGTGTGTTCACCTTTGACCCAGGCTTCACTTCAACCGCATCCTGCGAATCTAAAATTACTTTTATTGATGG
        410       420       430       440       450       460       470       480       490

 D  E  G  I  L  L  H  R  G  F  P  I  D  Q  L  A  T  D  S  N  Y  L  E  V  C  Y  I  L  L  N
TGATGAAGGTATTTTGCTGCACCGCGGTTTCCCGATCGATCAGCTGGCGACCGATTCTAACTACCTGGAAGTTTGTTACATCCTGCTGAA
        500       510       520       530       540       550       560       570       580

 G  E  K  P  T  Q  E  Q  Y  D  E  F  K  T  T  V  T  R  H  T  M  I  H  E  Q  I  T  R  L  F
TGGTGAAAAACCGACTCAGGAACAGTATGACGAATTTAAAACTACGGTGACCCGTCATACCATGATCCACGAGCAGATTACCCGTCTGTT
        590       600       610       620       630       640       650       660       670

 H  A  F  R  R  D  S  H  P  M  A  V  M  C  G  I  T  G  A  L  A  A  F  Y  H  D  S  L  D  V
CCATGCTTTCCGTCGCGACTCGCATCCAATGGCAGTCATGTGTGGTATTACCGGCGCGCTGGCGGCGTTCTATCACGACTCGCTGGATGT
        680       690       700       710       720       730       740       750       760

 N  N  P  R  H  R  E  I  A  A  F  R  L  L  S  K  M  P  T  M  A  A  M  C  Y  K  Y  S  I  G
TAACAATCCTCGTCACCGTGAAATTGCCGCGTTTCGCCTGCTGTCGAAAATGCCGACCATGGCCGCGATGTGTTACAAGTATTCCATTGG
        770       780       790       800       810       820       830       840       850

 Q  P  F  V  Y  P  R  N  D  L  S  G  Y  A  G  N  F  L  N  M  M  F  S  T  C  E  P  Y  E  V
TCAGCCATTTGTTTACCCGCGCAACGATCTCTCCTACGCCGGTAACTTCCTGAATATGATGTTCTCCACGCCGTGCGAACCGTATGAAGT
        860       870       880       890       900       910       920       930       940

 N  P  I  L  E  R  A  M  D  R  I  L  I  L  H  A  D  H  E  Q  N  A  S  T  S  T  V  R  T  A
TAATCCGATTCTGGAACGTGCTATGGACCGTATTCTGATCCTGCACGCTGACCATGAACAGAACGCCTCTACCTCCACCGTGCGTACCGC
         950       960       970       980       990      1000      1010      1020      1030

 G  S  S  G  A  N  P  F  A  C  I  A  A  G  I  A  S  L  W  G  P  A  H  G  G  A  N  E  A  A
TGGCTCTTCGGGTGCGAACCCGTTTGCCTGTATCGCAGCAGGTATTGCTTCACTGTGGGGACCTGCGCACGGCGGTGCTAACGAAGCGGC
       1040      1050      1060      1070      1080      1090      1100      1110      1120

 L  K  M  L  E  E  I  S  S  V  K  H  I  P  E  F  V  R  R  A  K  D  K  N  D  S  F  R  L  M
GCTGAAAATGCTGGAAGAAATCAGCTCCGTTAAACACATTCCGGAATTTGTTCGTCGTGCGAAAGACAAAAATGATTCTTTCCGCCTGAT
       1130      1140      1150      1160      1170      1180      1190      1200      1210

 G  F  G  H  R  V  Y  K  N  Y  D  P  R  A  T  V  M  R  E  T  C  H  E  V  L  K  E  L  G  T
GGGCTTCGGTCACCGCGTGTACAAAAATTACGACCCGCGCGCCACCGTAATGCGTGAAACCTGCCATGAAGTGCTGAAAGAGCTGGGCAC
       1220      1230      1240      1250      1260      1270      1280      1290      1300

 K  D  D  L  L  E  V  A  M  E  L  E  N  I  A  L  N  D  P  Y  F  I  E  K  K  L  Y  P  N  V
GAAGGATGACCTGCTGGAAGTGGCTATGGAGCTGGAAAACATCGCGCTGAACGACCCGTACTTTATCGAGAAGAAACTGTACCCGAACGT
       1310      1320      1330      1340      1350      1360      1370      1380      1390
```

```
 D  F  Y  S·G  I  I  L  K  A  M  G  I  P  S  S  M  F  T  V  I  F  A  M  A  R  T  V  G  W
GGATTTCTACTCTGGTATCATCCTGAAAGCGATGGGTATTCCGTCTTCCATGTTCACCGTCATTTTCGCAATGGCACGTACCGTTGGCTG
      1400      1410      1420      1430      1440      1450      1460      1470      1480

 I  A  H  W  S  E  M  H  S  D  G  M  K  I  A  R  P  R  Q  L  Y  I  G  Y  E  K  R  D  F  K
GATCGCCCACTGGAGCGAAATGCACAGTGACGGTATGAAGATTGCCCGTCCGCGTCAGCTGTATACAGGATATGAAAAACGCGACTTTAA
      1490      1500      1510      1520      1530      1540      1550      1560      1570

 S  D  I  K  R
AAGCGATATCAAGCGTTAATGG---3'
        1580      1590
```

Fig.5.1 Sequence of *glt*A and predicted protein sequence of *E.coli* citrate synthase (Ner *et al.*, 1983)

The single letter amino acid code is as follows: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

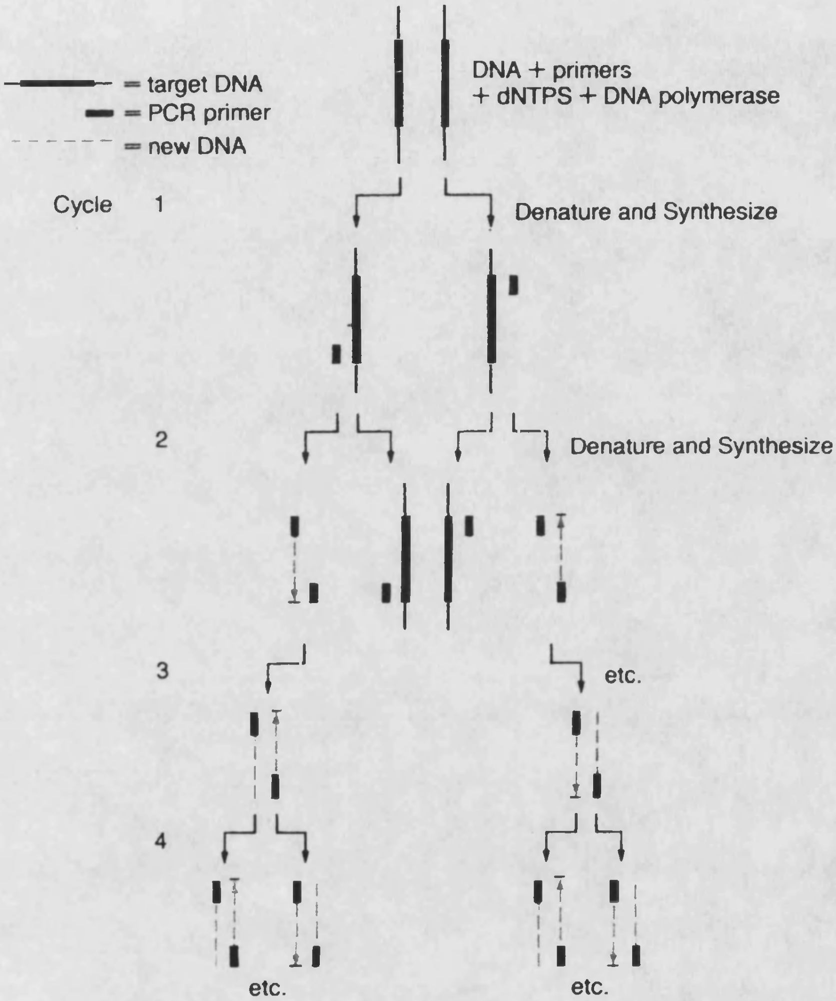
containing engineered restriction sites. The inclusion of restriction sites in the primers allows the fragment to be cloned directly into a suitable vector and by using different restriction sites, ligation of multiple fragments prior to insertion is prevented. The amplified fragment was then to be cut using the appropriate restriction endonucleases and inserted into the high copy number plasmid pUC18 (Yanisch-Perron *et al*, 1985; Messing, 1983) cut with the same enzymes.

pUC18 carries the *lacZ* gene which is induced by IPTG and is capable of α-complementation in *lacZ* mutants (Ullman *et al.*, 1967), such as TG1, giving rise to blue colonies when grown on plates containing the chromogenic substrate X-gal (Horwitz *et al.*, 1964). The *lacZ* gene of pUC 18 contains a multiple cloning site and insertion of DNA into this site disrupts the *lacZ* gene, abolishes complementation and gives rise to white colonies on X-gal plates.

5.3 DNA Amplification

The polymerase chain reaction (PCR) is a powerful technique based on the enzymatic amplification of specific regions of DNA from small amounts of complex material (Saiki *et al*, 1985). Repeated cycles of heat denaturation of the target DNA, annealing of primers flanking the segment of interest and extension of the annealed primers can result in the production of several million copies of the desired region (Fig. 5.2).

Although a relatively new technique, PCR has been used extensively in the study of eukaryotic systems and to some extent in prokaryotic systems. The specificity of the amplification reaction depends upon the sequence homology between the oligonucleotide primers and the target region of DNA. As the complete sequence of the *E.coli gltA* gene is known (Ner *et al.*,


= target DNA
= PCR primer
= new DNA
DNA + primers
+ dNTPS + DNA polymerase
Cycle 1
Denature and Synthesize
2
Denature and Synthesize
3
etc.
4
etc.
etc.

1983), DNA amplification was an ideal technique to use in order to isolate and amplify the mutant *glt*A gene.

Two oligonucleotide primers, complementary to regions of the *glt*A gene, were made (Method 3.26). The most reliable amplification has been shown to be achieved with non G:C rich primers of at least 20 bases (Shyamala & Ames, 1989) and this was, therefore, taken into account when designing the primers to use for amplification.

Each primer was 21 bases in length and contained two bases mismatched with the *glt*A gene to give an *EcoR* I site in one and a *Hind* III site in the other. The DNA segment between and including the primers consisted of 1 814 nucleotides and contained both putative CS promotor regions (Wilde & Guest, 1986) (Fig. 5.3).

Following 30 cycles of amplification of K114r4 chromsomal DNA (Method 3.27), one tenth of the sample was analysed by agarose gel electrophoresis (Method 3.13) (Fig. 5.4). A single product of the expected size (1.8 kb) was formed.

5.4 Cloning the 1.8 kb Amplified Product

50µl of the amplified mix was passed down a Sepharose CL6B (Pharmacia LKB) spun column (Method 3.22) to remove excess primers, dNTPs, which may interfere with ligation (Higuchi, 1989). The 3′ ends of the product were then end-filled using Klenow (Method 3.28), and after stopping the reaction, the sample was phenol:chloroform extracted (Method 3.10) to remove the enzyme and passed down another Sepharose CL6B (Pharmacia LKB) spun column (Method 3.22) to remove the phenol. The product was then digested (Method 3. 16) using *Hind* III and *EcoR* I and passed down a final Sepharose CL6B (Pharmacia LKB) spun column

EcoR1

5´---ATCATTA**G**AATT**C**ACCTACAT---3´

5---GCAGGGTGTTCCGGAGACCTGGCGGCAGTATAGGCTGTTCACAAAATCATTACAATTAACCTACATATAGT

TTGTCGGGTTTTATCCTGAACAGTGATCCAGGTCACGATAACAACATCAGGTCACGATAACAACATTTATTTAAT

TA rich

TAATCATCTAATTTGACAATCATTCAACAAAGTTGTTACAAACATTACCAGGAAAAGCATATAATGCGTAAAAGT

region recognition 1 Pribnow Box 1 S1↑

TATGAAGTCGGTATTTCACCTAAGATTAACTTATGTAACAGTGTGGAGTATTGACCAATTCATTCGGGACAGTTA

recognition 2

TTAGTGGTAGACAAGTTTAATAAATTCGGATTGCTAAGTACTTGATTCGCCATTTATTCGTCATCAATGGATCCT

Pribnow Box 2 S2↑

TTACCTGCAAGCGCCCAGAGCTCTGTACCCAGGTTTTCCCCTCTTTCACAGAGCGGCGAGCCAAATAAAAACGGG

TAAAGCCAGGTTGATGTGCGAAGGCAAATTTAAGTTCCGGCAGTCTTACGCAATAAGGCGCTAAGGAGACCTTAA

ribosome binding site

M A D T K A K

ATGGCTGATACAAAAGCAAAA

E K R D F K S D I K R _

5´---GAAAAACGCGACTTTAAAAGCGATATCAAGCGTAATGGTTCGATTGCTAAGTTGTAAATATTTTAACCC

Hind III

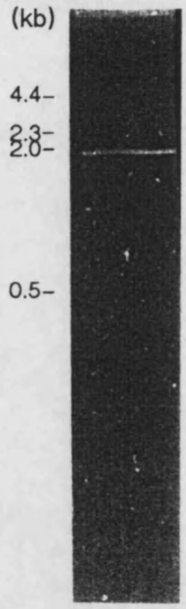
3´---TATACCG**TT**CGAACTAAAAATA---5´

GCCGTTCATATGGCGGGCTTGATTTTTATA---3´

Fig. 5.3. Oligonucleotides used for DNA amplification.

(Mismatches shown in bold type. ↑ indicates initiation of transcription.)

Fig. 5.4. Agarose gel electrophoresis of a 1.8 kb fragment of the *glt* A gene generated by DNA amplification of chromosomal DNA from K114r4. Size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.


(kb)
4.4-
2.3-
2.0-
0.5-

(Method 3.22) before ligation (Method 3.29) into *Hind* III-*EcoR* I cut pUC18. The ligated DNA was then transformed (Method 3.31) into freshly-prepared competent TG1 cells (Method 3.30). 200µl aliquots of transformed cells were then mixed with X-gal and IPTG (Method 3.2), spread onto LB agar plates and incubated overnight. On the basis of colour selection, 49 recombinants were obtained.

5.5 Identification of Recombinants

10 white colonies were picked, from which plasmids were purified by mini-preparation (Method 3.23) and then digested (Method 3.16) with *Hind* III and *EcoR* I restriction enzymes. Agarose gel electrophoresis (Method 3.13) of the digests is shown in Fig. 5.5. All the colonies picked contained a recombinant plasmid, 9 of which when cut with *Hind* III and *EcoR* I gave two fragments: one of approximately 2.6 kb corresponding to the pUC 18 plasmid and one of approximately 1.8 kb corresponding to the amplified fragment of the *glt*A gene. The fragment observed in the tenth clone has an insert of approximately 3.6 kb, maybe corresponding to the ligation of two 1.8 kb fragments although every precaution was taken to prevent this happening. To ensure that the 1.8 kb fragment was from the *glt*A gene, single digests (Method 3.16) of one of the plasmid preparations were prepared using *Bgl* I and *Pvu* II restriction enzymes and analysed by agarose gel electrophoresis (Method 3.13). These restriction endonucleases were chosen for simplicity as they recognise only a few sites on pUC 18 and the *glt*A gene. The results are shown in Fig. 5.6. The fragments generated were of the expected size indicating that the correct fragment had been amplified and cloned.

Fig. 5.5. The 1.8 kb amplified *glt* A fragment was ligated together with *Hind* III-*Eco* RI cut pUC18 and transformed into *E.coli* TGI. Mini-preparations of plasmid DNA were prepared from a selection of the resultant clones and the DNA was digested with *Hind* III and*EcoR* I. (Lane: 1. pUC 18 digested with *Hind* III-*EcoR* I; 2-11. recombinants). Size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.


(kb)
1
2
3
4
5
6
7
8
9
10
11
4.4–
2.3–
2.0–
0.5–

Predicted sizes of fragments generated by *Bgl* I and *Pvu* II digestion of a recomninant containing a 1.8 kb amplified fragment of the *glt* A gene. Sizes are shown in kb

Fig. 5.6. Agarose gel electrophoresis of *Bgl* I and *Pvu* II digests of one recombinant obtained from ligation of a 1.8 kb amplified fragment of the *glt* A gene with pUC18. Size and position of fragments generated by *Hind* III digestion of λ DNA are shown on the left hand side.

*Bgl*I	*Pvu*II
2723	2364
1118	1000
609	674
	228
	184

The DNA amplification and cloning procedures were then repeated using chromosomal DNA from *E.coli* K114 and DNA prepared by maxi-preparation (Method 3.24) and caesium chloride density gradient (Method 3.25) of the plasmid pDB2 (the *glt*A gene cloned into pBR322 (Bloxham *et al.*, 1983)). All amplifications and transformations were carried out on separate occasions to guard against cross-contamination. 20 white colonies were obtained from K114 and 37 from pDB2. Again a sample of recombinants (6) from each cloning experiment were digested (Method 3.16) with *Hind*III and *EcoR*I and on examination on agarose gels (Method 3.13) were all found to contain DNA inserts of approximately 1.8 kb.

5.6 Sequence Strategy

Initially, DNA from one of each type of recombinant plasmid was purified by maxi-preparation (Method 3.24) and purified further by caesium chloride density gradient (Method 3.25). Single stranded templates were made (Method 3.32) and the DNA sequenced (Method 3.33) in both directions using primers made to both the sense and anti-sense DNA strands.

In some instances, DNA amplification has been shown to introduce errors in the DNA sequence due to lack of fidelity of the *Taq* polymerase (Saiki *et al.*, 1988; Tindall & Kunkel, 1988; Keohavong & Thilly, 1989; Reiss *et al.*, 1990). Thus, when sequencing individual clones of DNA-amplified fragments, any point mutations observed could be just one of these errors. To overcome this problem, any region found to contain a mutation was re-sequenced in a further two transformants, as the same error would be unlikely to occur in three different clones.

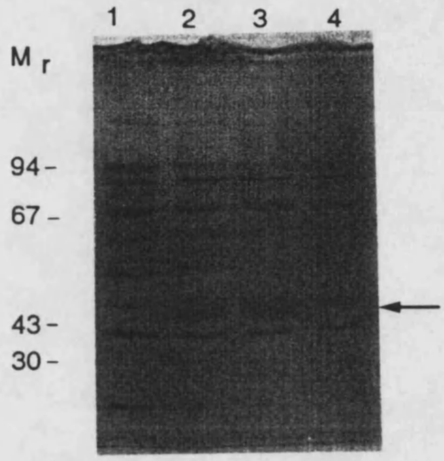
5.7 Sequence Analysis

When the wild type clone was sequenced, it proved to match the published sequence of the *gltA* gene (Ner *et al.*, 1983) exactly and included the 1170 T to G correction as described by Anderson and Duckworth (1987). The frequency of nucleotide misincorporation under the conditions used here is,therefore, less than 1 in 1 814 bp, a result which compares with those studies also finding low misincorporation (Scharf *et al.*, 1986; Loh *et al.*, 1989; Shyamala & Ames, 1989). The K114 *gltA* clone was found to contain a single point mutation, G to A at position 1392, resulting in the replacement of aspartate 362 by asparagine. The same base change was seen in a further two clones from the K114 amplification. When the K114r4 *gltA* clone was sequenced a single base change was also observed which was exactly the same as that found in the K114 clone. Two further K114r4 clones were sequenced in this region and they too contained this base change. As a further precaution, a second DNA amplification of K114r4 was performed and the amplified fragment cloned as before (5.4). Three of the resulting clones were sequenced in the region containing the base change and they too contained the same point mutation.

5.8 Expression of the Cloned *gltA* genes

TG1 and the TG1 clones containing the *gltA* gene from K114, K114r4 and wild-type *E.coli* were grown overnight in 100ml LB containing 100mg ampicillin/ml broth. 1.5 ml of the culture were lysed using the rapid lysis technique (Method 3.35) and cell extracts were analysed by SDS-PAGE (Method 3.36) (Fig. 5.7). Cell free extracts (Method 3.2) were made from the remaining culture and assayed for CS activity (Method 3.3). The levels of CS in the three transformants were compared with those obtained for TG1

Fig. 5.7. Cell-free extracts of 1. *E.coli* TG1 and *E.coli* TG1 containing *glt* A from 2. K114r4, 3. K114, 4. pDB2 were run on an SDS PAGE gel. The expression of a protein of approximate subunit M_r 45 000 is indicated by the arrow. The relative molecular mass and position of standard proteins are shown on the left hand side.


1
2
3
4
M r
94 -
67 -
43 -
30 -

alone. Clones of the 1.8 kb fragment from K114 and K114r4 gave specific activities of CS of 0.220 U/mg protein and 0.228 U/mg protein respectively which is comparable with that from TG1 alone, 0.243 U/mg protein. However, CS activity in the wild type clone was 0.657 U/mg protein which represents a 3 fold amplification compared with the CS activity of TG1. This amplification factor is similar to that reported for other CS clones (Guest, 1981).

5.9 Complementation of W620

W620, a strain of *E.coli* lacking CS activity (Reissig & Wollman, 1963), was transformed (Method 3.31) with the K114, K114r4 and wild type recombinant plasmids generated above (5.4). As a control the plasmid pDB2 was also used to transform the cells. Transformants were first selected for their ability to grow on LB in the presence of ampicillin (20μg ampicillin/ml LB) and then for their ability to grow on succinate minimal medium in the absence of glutamate (4.2). As W620 has been shown to revert and regain CS activity fairly easily (Else, 1986) it was grown as a control on succinate minimal medium plates to distinguish true complementation from reversion.

All transformants grew in the presence of ampicillin indicating that transformation had been successful. Colonies appeared on the succinate minimal medium plate of W620 containing pDB2 after 48 h, consistent with that previously reported (Bloxham *et al.*, 1983). W620 containing the amplified wild type *glt*A gene also grew on succinate minimal medium plates but was slower to grow than that containing pDB2 (72h), maybe as a result of the lower expression of CS from this construct compared with pDB2 (Bloxham *et al.*, 1983). However, there was no evidence of growth on plates containing untransformed W620 and so it can be assumed that the amplified wild type *glt*A gene was complementing the W620. W620 containing the *glt*A gene from either K114 or K114r4 failed to grow on succinate minimal medium plates indicating that the CS produced by this gene was unable to compensate for the defect in the W620 citric acid cycle.

5.10 Discussion

The *glt*A gene that encodes CS was sequenced from K114r4, a mutant of *E.coli* that produces CS with altered catalytic and structural properties. The sequence was compared with that of the *glt*A genes from a wild type*E.coli* strain and from K114, the parent of K114r4, in order to investigate any modifications at the molecular level and thus, gain some insight into the diversity of structure-function relationships displayed by CSs.

DNA amplification was used to isolate the *glt*A gene from chromosomal and plasmid DNA. A single product of the anticipated size was amplified indicating the high specificity of this technique under the conditions used.

The cloning strategy used proved to be successful, producing many white colonies. On analysing a sample of these all contained inserts, 95% of which were of the correct size. Restriction digests of one of these recombinant plasmids generated fragments of the expected size confirming that the *glt*A gene had been amplified and cloned successfully.

Some workers have reported that DNA amplification results in a high frequency of misincorporation errors (Scarf *et al.*, 1986; Loh *et al.*, 1989; Shyamala & Ames, 1989). When the amplified wild type clone was sequenced, however, the sequence of nucleotides was found to be identical to that published. Given the low error frequency observed, DNA amplification was a reliable technique to use as part of the strategy to identify the minor nucleotide sequence differences between K114r4 *glt*A gene and the wild type *glt*A gene.

When the *glt*A gene from the mutant K114r4 was sequenced, a single mutation was found, namely, the conversion of a G to A at position 1392 resulting in the replacement of Asp 362 with Asn 362. Interestingly, sequence analysis of the *glt*A gene from K114, the parent of K114r4, showed this same base substitution.

Sequence homology has shown that this residue, equivalent to Asp 375 of pig heart CS, is highly conserved throughout all CSs sequenced so far. Moreover, Asp 362 (and equivalent residues) has been implicated, from both crystallographic and site-directed mutagenesis studies as a key residue involved in the catalytic mechanism of CS (Wiegand & Remington, 1986; Handford *et al.*, 1988; Evans *et al.*, 1989; Kispal *et al.*, 1989; Alter *et al.*, 1990; Karpusas *et al.*, 1990; Karpusas *et al.*, 1991).

The course of catalysis by CS can be divided into three consecutive partial reactions; enolization, condensation, and hydrolysis. It is proposed that Asp 362 is involved in both the enolization and hydrolysis steps of the reaction sequence. In the enolization, Asp 362 acts in concert with His 264 in general acid-base catalysis. His 264 acts as an acid, donating a proton to the carbonyl oxygen of the acetyl group of the acetyl-CoA, whilst Asp 362 acts simultaneously as a base, accepting a proton from the methyl group of acetyl-CoA, resulting in the formation of a neutral enol intermediate. Condensation of the enol intermediate with OAA is accomplished, again by acid-base catalysis. His 305 acts as the acid this time, protonating the carbonyl oxygen of OAA whilst His 264 accepts a proton from the hydroxyl of the enol intermediate as it condenses with OAA forming a cityl-CoA .CS complex. Thus, the proton is recycled, restoring His 264 to the charge state these results could only be explained if during the course of evolution the *Hind* III and *EcoR* I sites had remained intact and there had been no others introduced but that the regions homologous to the DNA amplification probes has undergone changes such that the probes no longer bound. Further characterisation and confirmation that this altered CS is distinct from the *glt*A gene product is required in order to investigate the nature of the CS displayed by K114r4.

6. IMMUNOLOGICAL STUDIES ON THE MUTANT CS FROM K114r4

6.1 Introduction

A mutant of *E.coli* has been created that produces a CS with altered kinetic, regulatory and structural properties compared with the wild type enzyme. On cloning and sequencing the *glt*A gene that encodes for *E.coli* CS a single mutation was found that caused the replacement of Asp 362 with Asn 362. Asp 362 has been implicated as one of the essential residues of CS for catalysis, participating in both enolization of acetyl-CoA and cleavage of citryl-CoA (Remington *et al.*, 1982; Karpusas *et al.*, 1990; Karpusas *et al.*, 1991). Indeed, site directed mutagenesis involving replacement of Asp 362 with several amino acids, including Asn, has shown, that without Asp 362, CS is inactive (Handford *et al.*, 1988; Evans *et al.*, 1989; Alter *et al.*, 1990). This was paralleled in this work as no increased activity above the base level was observed in bacteria transformed with plasmid containing the K114r4 *glt*A gene. However, K114r4 does display CS activity and, therefore, as the *glt*A gene appears to have been inactivated it is proposed that the protein displaying CS activity must be the product of another gene.

Although, most of the proteins of *E.coli* are of a single molecular species encoded by a single gene, there are many instances where two or more genes encode for enzymes catalysing the same reaction (Bachman, 1990). These multiple genes may be either duplications of the same gene resulting in the production of closely related proteins or may have arisen independently by convergent evolution (Zipkas & Riley, 1975).

A probe made to the 3.2 kb *Hind*III-*EcoR*I fragment carrying the *glt*A gene only hybridised with one fragment of 3.2 kb after restriction digest and Southern blotting of K114r4 chromosomal DNA (4.5). It is, therefore, proposed that the gene encoding the altered CS is distinct from the *glt*A gene and that it produces a protein which may be structurally unrelated to wild-type CS. To test this hypothesis a polyclonal antibody was raised against wild type *E.coli* CS and used in immunological studies of *E.coli* strains K114, K114r4 and HB101.

6.2 <u>Purification of Wild Type *E.coli* CS</u>

Wild type *E.coli* CS was purified from the organism DB1002 (Bloxham *et al.*, 1983) using a previously developed procedure (Else, 1986) which is summarised in Table 6.1. DB1002 is the CS deficient strain of *E.coli*, W620, transformed with the plasmid pDB2 which contains the *glt*A gene.

2.2g (wet weight) of cells from 500ml of an overnight culture of DB1002 in LB containing 100μg ampicillin/ml broth were used for the enzyme purification. Following treatment with protamine sulphate (Method 3.37), the extract was applied to a column (30cm x 3cm) of DEAE Sephadex (Pharmacia LKB) equilibrated with 20mM Tris/HCl pH 8.0, 1mM EDTA. The CS was eluted with a linear gradient of 0 to 0.5M NaCl in 500ml buffer. The flow rate was 15ml/h, and 3ml fractions were collected. Fractions containing CS with specific activity greater than 20U/mg protein were pooled, and the buffer changed to 20mM triethanolamine (TEA) pH 7.3, 1mM EDTA using a PD10 column (Pharmacia LKB). The sample was then applied to a Mono Q ion exchange column (Pharmacia LKB) connected to an FPLC system (Pharmacia LKB). The bound CS was eluted with a linear gradient of 0 to 0.5M NaCl in 50 ml of 20mM TEA pH 7.3, 1mM EDTA. The flow rate was 1ml/min and 1ml fractions were collected. Fractions with a specific activity greater than 45U/mg protein were pooled and desalted using a PD10 column (Pharmacia LKB). The sample was then re-applied to the Mono Q column and eluted as before.

	VOLUME (ML)	TOTAL CS (U)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %
SONIC EXTRACT	5.4	224	68	3.3	
DEAE COLUMN	5	137	5.5	25.1	60
FIRST MONOQ	1	78	1.6	49.0	56
SECOND MONO Q	0.5	40	0.5	79.7	51

Table 6.1. Summary of Purification of Wild-Type CS

The CS recovered from the final step of the purification had a specific activity of 79.7 U/mg protein, which represents a 24-fold purification of the cell free extract. This specific activity obtained is similar to that reported from previous preparations of pure plasmid-encoded CS, using this method (Else, 1986) and others (Duckworth & Bell, 1982; Bloxham *et al.*, 1983; Robinson *et al.*, 1983).

Electrophoresis of the purified CS on SDS-PAGE (Method 3.36) gave a single band corresponding to a sub-unit M_r~45 000 (Fig. 6.1) confirming its homogeneity.

6.3 Preparation of a Polyclonal Antiserum Against Wild Type *E.coli* CS

The purified wild type *E.coli* CS was used to raise a polyclonal antiserum in a rabbit (Method 3.39). Prior to immunisation, a 10ml test bleed was taken from the rabbit to act as a negative control. A final bleed was made on day 35 and the serum (Method 3.40 from this was used in the immunological studies.

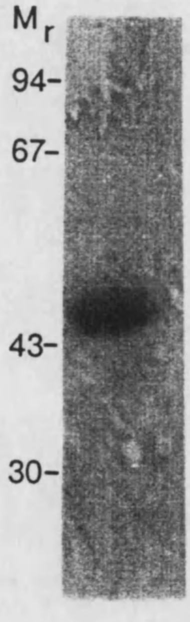
6.4 Characterisation of the Antiserum

6.4.1 Inhibition of CS

As polyclonal antibodies recognise multiple epitopes on the surface of the antigen, they frequently cause inhibition of the enzyme to which they have been raised. This fact was used to monitor the binding of the antibody to the wild type and mutant enzymes (6.5).

10µl of a cell free extract (Method 3.2) from *E.coli* HB101, was incubated with either pre- or post-immune serum, both of which were diluted 500-fold in mM Tris/HCl pH 8.0 for 5 min. The sample was then assayed for CS activity (Method 3.2) and compared with the activity of HB101 CS in the

Fig. 6.1. SDS-PAGE of Purified Wild-Type CS


M r
94-
67-
43-
30-

absence of serum. The pre-immune serum (160 μg/ml assay mixture) did not effect the activity of HB101 CS whereas the activity decreased from 0.30 U/mg protein to 0.019 U/mg protein in the presence of post-immune serum (130 μg/ml assay mixture) which represents a 94% inhibition of CS activity.

6.4.2 Immunoblotting

Crude cell extracts (Method 3.2) of HB101 were run on SDS-PAGE (Method 3.36) and transferred to nitrocellulose by Western blotting (Method 3.42). After blocking non-specific sites, the filters were incubated with either pre- or post-immune serum diluted 1000-fold. Hybridisation was detected using a horseradish peroxidase conjugated anti-rabbit IgG (Method 3.43) followed by chromographic development (Method 3.43).

The post immune serum recognised a single protein band of M_r-45 000 in cell free extract (Fig. 6.2). No immunoreactivity was observed with pre-immune serum (Fig. 6.2).

6.4.3 Sensitivity

Samples of decreasing amounts of the purified wild type CS were run on SDS-PAGE (Method 3.36) and immunoblotted (Method 3.42). The post-immune serum was able to detect 5ng of CS (Fig. 6.3).

When cell free extracts of TG1 transformed with K114r4 were run on SDS-PAGE, the mutation appeared to have no effect on the production of CS (5.8). Therefore, it seemed inappropriate to investigate the immunoreactivity of the mutant protein by Western blot as results would be hampered by the presence of contaminating wild type enzyme. It was, therefore, decided to investigate antibody binding of the mutant protein after

Fig. 6.2. Western Blot of cell free extracts of *E.coli* DB1002 with polyclonal antiserum raised against CS.

(Lane 1. Purified CS; 2. Cell extract; 3. pre-immune serum; 4. post-immune serum)

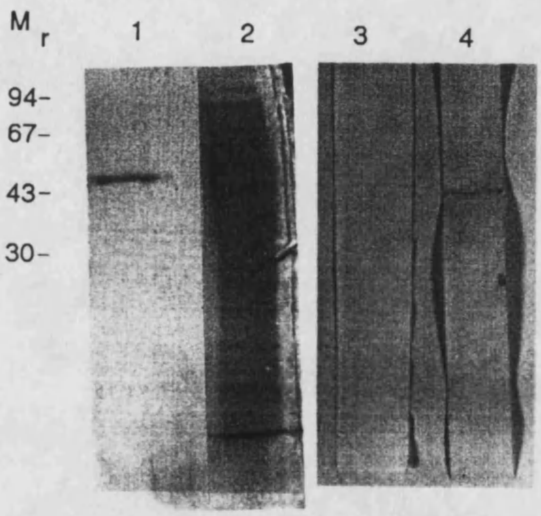
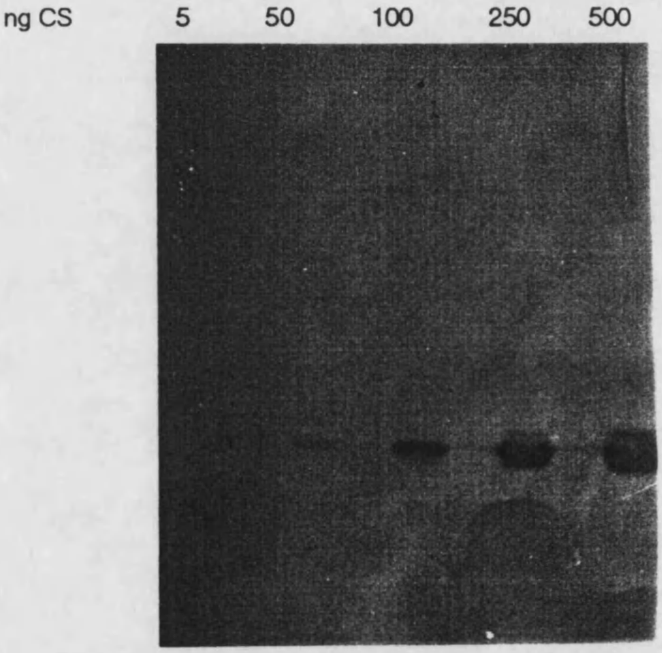

M r
1
2
3
4
94-
67-
43-
30-

Fig. 6.3. Sensitivity of the Polyclonal Antiserum. Varying amounts of purified *E.coli* CS were Western Blotted and reacted with a polyclonal antiserum raised against wild-type *E.coli* CS.


ng CS
5
50
100
250
500

removing the 'large' wild type enzyme by gel filtration.

Gel filtration of 200µl cell free extract (Method 3.2) of K114r4 was carried out using a Superose 12 gel filtration column (Pharmacia LKB) attached to an FPLC system (Pharmacia LKB) (Method 3.8). 3µl aliquots from each fraction were then applied to duplicate nitrocellulose filters by the dot blot method (Method 3.41). The filters were then reacted with either pre- or post-immune serum (Method 3.43) and immuno-recognition determined using a horseradish peroxidase conjugated anti-rabbit IgG (Sigma) and chromogenic development (Method 3.43). The procedure was then repeated using cell free extracts from HB101 and K114.

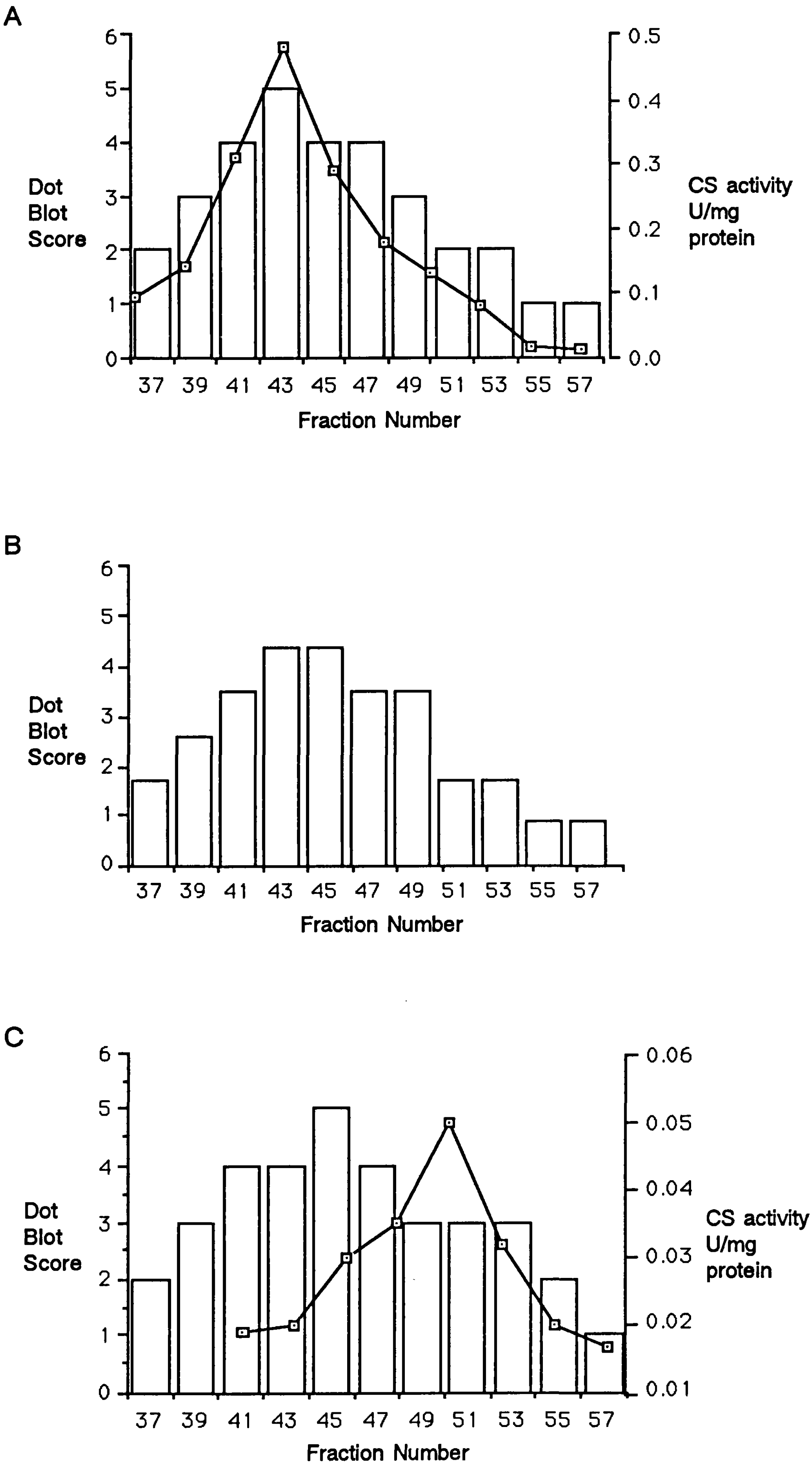
No reaction was seen when the filters were reacted with the pre-immune serum. Fig. 6.4A shows the HB101 CS elution profile and dot blot score. Peak CS activity was observed in fraction 43 (0.48 U/mg protein) and on analysis, immunoreactivity of the fractions mirrored CS activity as expected.

Predictably, K114 did not display CS activity in fractions eluted from the Sepharose 12 column. However, the immunoblot gave positive results in a distribution that matched that of HB101 (Fig. 6.4B). The elution of CS from K114r4 was also as expected in that the peak of CS activity (Fraction 51) eluted after that from wild type *E.coli* (Fig. 6.4C). However, the peak of antibody binding (Fraction 45) did not coincide with the peak of enzyme activity but was more consistent with the results obtained from the wild type CS.

6.5 Inhibition of K114r4 CS activity

The ability of the polyclonal antiserum to inhibit CS activity from K114r4 was investigated as described above (6.4.1) No detectable

Fig. 6.4. Relationship Between Elution profile of CS from Superose 12 column and antibody binding (histogram). Cell free extracts of *E.coli* A. TG1; B. K114 and C. K114r4 were applied to a Superose 12 gel filtration column. Fractions were assayed for CS and dot blotted. The dot blot was reacted with antiserum raised against wild-type *E.coli* CS and the intensity of the reaction of each dot scored on a scale of 1 to 5, 5 being the strongest reaction.


A
Dot Blot Score
CS activity U/mg protein
Fraction Number
B
Dot Blot Score
Fraction Number
C
Dot Blot Score
CS activity U/mg protein
Fraction Number

decrease in activity was detected in the presence of the anti-CS polyclonal antibody (130 μg/ml assay mixture).

6.6 Discussion

Pre-immune serum did not bind to any *E.coli* proteins indicating that this could be used with confidence as a negative control and that any reaction seen with the post-immune serum was due to antibody raised against *E.coli* CS. The polyclonal antibody produced showed high specificity for CS, recognising only that protein in a complex mixture of proteins such as that found in crude cell extracts. Furthermore, the antiserum was able to detect as little as 5ng of purified *E.coli* CS on a Western blot.

When fractions were taken from gel filtration of a cell free extract of K114 and reacted with the CS antiserum, it was shown, that although K114 does not display CS activity, it still produces the protein which eluted from the column in a similar volume to the CS from the wild type *E.coli*. This is consistent with the sequence data which showed that the promotor sequence of the K114 *glt*A gene was unaltered and that the only change involved the active site residue Asp 362 which is essential for activity. Previous work (Alter *et al.*, 1990) has shown that altering the equivalent residue in the pig heart enzyme does not effect binding of an anti-PHCS monoclonal antibody family.

When fractions from the gel filtration of a cell free extract of K114r4 were analysed, the peak CS activity did not correspond to the peak of antibody binding. The peak antibody binding was observed in fractions corresponding to those where wild-type CS activity is normally observed. It would appear that as in K114, the normal wild-type hexameric enzyme is being produced but is inactivated due to the nucleotide substitution. The broad peak of antibody binding reflects the elution of the wild type enzyme. No second peak of antibody binding was observed in those fractions containing the mutant CS activity. The lack of positive reaction on the dot blot together with the fact that the post-immune serum did not inhibit the activity of the mutant protein suggests that the mutant protein is distinct from the wild type protein

Likewise, other mutants of CS have been shown to be unaffected by antiserum raised against the wild type enzyme. Weitzman *et al.* (1978) generated a 'small' NADH insensitive mutant from *A.anitratum* which was not inactivated by wild type antiserum. Similarly no immuno-cross-reactivity was observed between a 'small' NADH insensitive CS mutant from *P.aeruginosa* and the wild type enzyme (C. Mitchell, personal communication).

The results from this and the previous chapters show that a mutant of *E.coli* capable of growth in the absence of glutamate has been created from a previously CS deficient strain unable to grow without glutmate supplementation. This mutant produces a protein that is capable of catalysing the same reaction as CS but displays different kinetic and molecular properties compared to the wild type enzyme, differs immunologically from the native CS and is not encoded by the *glt*A gene. The question then arises as to the nature of this mutant protein.

7. PURIFICATION AND N-TERMINAL SEQUENCE OF THE MUTANT PROTEIN.

7.1 Introduction

By mutating K114, a strain of *E.coli* deficient in CS activity, revertants were selected that had regained CS activity. The majority of these revertant CSs displayed wild type characteristics but a few displayed altered properties. Further investigation of the CS from one of these revertants, K114r4, revealed that it bore a striking resemblance to CS produced by eukaryotes, namely, a M_r~118 000, insensitivity to 2-oxoglutarate and NADH and high affinity for the substrates acetyl-CoA and OAA. It was assumed, that such altered properties were a result of second site mutations within the *glt*A gene, the structural gene for CS. However, sequence analysis revealed that the *glt*A gene of the revertant possessed only one mutation, which was the same mutation as that in the *glt*A gene of K114: the conversion of G to A at position 1392, resulting in the replacement of the key residue Asp 362 with Asn. Such a mutation has been shown to result in the loss of CS activity (Handford *et al,* 1988; Evans *et al.*, 1989; Kispal *et al.*, 1989; Alter *et al.*, 1990) and, indeed, the *glt*A gene of the revertant was unable to complement the CS deficient strain, W620. Therefore, as K114r4 possesses an enzyme that is capable of catalysing the condensation of OAA with acetyl-CoA it is proposed that the enzyme responsible for this reaction is not a product of the *glt*A gene. Furthermore, this mutant enzyme would appear to be not closely related to CS as it shows no immunological cross-reactivity with wild type CS antiserum. In order to go some way towards identifying the gene responsible for this mutant CS activity it was decided to purify the protein so that the N-terminal sequence could be determined. With this information it would then be possible to search protein data banks in the hope of identifying a homologous protein.

7.2 Purification of the Mutant CS from K114r4

A mutant *E.coli* CS displaying properties similar to those of the mutant protein in this study has been purified to near homogeneity (Robinson, 1984). Attempt to purify the mutant CS obtained in this study using this method failed to achieve the level of purification previously reported. The method employs a gel filtration step using Sephadex G200 (Pharmacia LKB) and it was at this stage that problems were encountered. The enzyme is very unstable and probably as a result of the dilution effects caused by gel filtration all activity was lost at this stage. Addition of glycerol, which normally improves stability of the enzyme had little effect, probably because of the increased run time due to the higher viscosity of the running buffer. It was decided, therefore, to adapt the method used for the purification of the wild type enzyme as this method uses ion exchange which is not hampered by such large dilution effects encountered in gel filtration.

A summary of the purification is presented in Table 7.1. 42.6g (wet weight) of cells from 8 litres of a 48 h culture of K114r4 in minimal medium were used for the purification. A cell free extract was prepared by sonication and the cell debris removed by centrifugation (Method 3.2). Nucleic acids were removed from the extract by treatment with protamine sulphate (Method 3.36). The sample was then applied to a column (30cm x 3cm) of DEAE-Sephadex (Pharmacia LKB) equilibrated with 20mM Tris/HCl pH 8.0, 1mM EDTA, 20% (v/v) glycerol at 4 C. The CS was eluted with a linear gradient of 0 to 0.5M NaCl in 500ml buffer. The flow rate was 15ml/min and 3ml fractions were collected. The peak fractions were pooled and desalted using a PD 10 column (Pharmacia LKB). The mutant protein did not bind to a Mono Q ion exchange column in triethanolamine pH 7.3, further indicating that it differs from the wild type enzyme. Instead, it was applied to the Mono

Q column in 20mM Tris/HCl pH 8, 1mM EDTA, 20% (v/v) glycerol. The CS was eluted by a linear gradient of 0 to 0.5M NaCl in 50 ml buffer. The flow rate was 1ml/min and 1ml fractions were collected on ice. The peak fractions were pooled and the buffer changed to 50mM MES/NaOH pH 5.5, 1mM EDTA, 20% (v/v) glycerol using a PD 10 column. The sample was applied to a Mono S ion exchange column (Pharmacia LKB) connected to an FPLC system (Pharmacia LKB). The bound CS was eluted using a linear gradient of 0 to 0.5M NaCl in 50 ml buffer. The flow rate and sample collection was as before.

The material recovered from the Mono S column had a specific activity of 1.94U/mg protein which represents a 62 fold purification of the mutant protein although large losses were incurred. 10% SDS-PAGE revealed two bands: a major band of M_r~45 700 and a minor band of M_r~50 100 (Fig. 7.1).

7.3 Immunoreactivity of the Mutant CS

As the mutant CS had been purified, the immunoreactivity of the protein by Western blotting was investigated. 500ng was loaded onto an SDS polyacrylamide gel, electrophoresed (Method 3.36), and Western blotted (Method 3.42). No immunoreactivity was observed with either band of the mutant CS whereas strong reactivity was seen with the wild type CS (20 ng) (Fig. 7.2).

7.4 N-Terminal Sequencing

7.4.1 Protein Blotting

20μg of the purified mutant CS was run on a 12.5% SDS polyacrylamide gel (Method 3.36) at 30mA until the first blue dye had

	VOLUME (ML)	TOTAL CS (U)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg)	YIELD %
SONIC EXTRACT	5.4	224	68	3.3	
DEAE COLUMN	5	137	5.5	25.1	60
FIRST MONOQ	1	78	1.6	49.0	56
SECOND MONO Q	0.5	40	0.5	79.7	51

Table 7.1. Summary of Purification of Mutant Protein from K114r4

Fig. 7.1. SDS-PAGE of the Purified Mutant Protein from K114r4

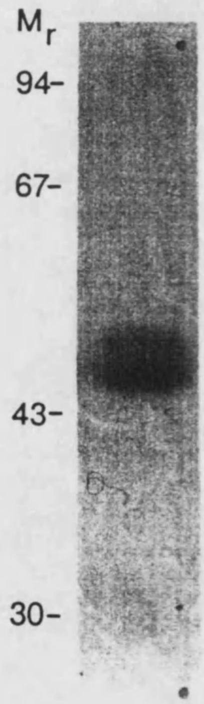

M_r
94-
67-
43-
30-

Fig. 7.4. Alignment of Citrate Synthase Sequences.

● residues implicated in catalysis.

Letters below the sequences refer to amino acids conserved in all sequences

Regions of secondary structure in the pig heart enzyme are indicated by a horizontal line above the sequence


Wild-Type CS
K114r4 CS

reached the bottom of the gel. The gel was then transferred to Immobilon PVDF (Millipore) by electrophoresis (Method 3.44). Following transfer the blot was stained by soaking in 0.025% (w/v) Coomassie Brilliant Blue, 50% (v/v) methanol, 10% (v/v) HAc for 15 min and destained by soaking in 50% (v/v) methanol, 10% (v/v) HAc for 30 min. The blot was stored at -20 °C until required.

7.4.2 Microsequencing

The blot, still wet, was sent to Imperial Chemical Industries (ICI), Pharmaceutical Division, for microsequencing. Sequencing was carried out on an Applied Biosystems 470A gas-phase sequencer coupled to an Applied Biosystems 120 phenylthiohydantoin analyser by Janice Young of the Biotechnology Department at ICI. Both bands were sequenced and the results are shown in Fig. 7.3. Both bands gave the same N-terminal sequence and it is assumed that they are products of the same protein. The two bands, therefore, probably represent different states of the protein with the band of lower molecular weight maybe arising due to degradation of the protein.

A search was made of protein databanks using the Seqnet vax computer at Daresbury. Two protein data bases were searched: the NBRF protein data base using the Fasta program and the SwissProt 17.0 data base. No proteins were found with N-terminal sequences that matched that of the mutant CS.

7.5 Discussion

The CS from K114r4 had been purified and the N-terminal portion sequenced. The mutant and wild type proteins displayed different binding

MAJOR BAND -

NSVALSGVPAGNTAL?TVGKSGNDL?Y(R)GY??LD(L)?K

MINOR BAND -

```
???ALSPAGN?A
   L V   S
```

Fig. 7.3. N-Terminal Sequences

properties to Mono Q ion exchange resin indicating that they have different charge properties. A Western blot of the purified mutant protein showed no reactivity towards an antiserum raised against the wild type CS suggesting that the proteins are not closely related.

Searches of protein data bases unfortunately did not reveal the identity of this mutant protein. However, this is probably not surprising as only 31.6% of the entire *E.coli* chromosome has been sequenced to date (Kroger *et al.*, 1991). Comparison of the N-terminal sequence obtained for the mutant protein with other CSs (Fig. 7.4) reveals no obvious similarity. However, the N-terminal portions of CSs show the least interspecies homology and, therefore, more sequence data are needed before any real comparisons can be made.

Fig. 7.2. Immunoreactivity of Wild-type CS and the Mutant Protein from K114r4 with a Polyclonal Antiserum Raised Against Wild-type CS

```
  1      10        20        30        40        50        60        70        80        90        100       110       120       130
1 --ASSTN--LKDILADLIPKEQARIKTFRQQH-GNTV-VGQI-TVDMMYGG------M-RGMKGLVY-E-TSVLDPD--EGIRF-RGY-SIPECQKMLPKAKGGEEPLPEGLFWLLVTGQI--P-TEEQV
2 --STDLD--LKSQLQELIPEHKDRLKKLKSEH-GK-VQLGNI-TVDMVIGG------M-RGMTGLLW-E-TSLLDPE--EVFAL--GDCRLPECQKALLPTAQSGG-L-NHYRRSFVAS-LNWKGTLAKS
3 --ASEQT--LKERFAEIIPAKAQEIKKFKKEH-GKTV-IGEVL-LEEQAYGG------M-RGIKGLVW-EGSVL---DPEEGIRF-RGR-TIPEIQRELPKAEGSTEPLPEALFWLLLTGEI--P-TDAQV
4 --ADTKAK-LT--LNGDTAVELDVLKGTLGQD----V-I-DIRTLGSKGVF---T--FDPGFTSTASCE-SKITFIDGDEGILLHRGF-PIDQLATDS--NY-----L-EVCYILLNG-EK--P-TQEQY
5 SEATGKKAVLH--LDGKEIELPIYSGTLGPD-----V-I-DVKDVLASGHF---T--FDFGFMATASCE-SKITFIDGDKGILLHRGY-PIDQLATQA--DY-----L-ETCYLLLNG-EL--P-TAEQK
6 --ADKKAQ-LI--IEGSAPVELPVLSGTMGPD----V-V-DVRGLTATGHF---T--FDPGFMSTASCE-SKITYIDGDKGVLLHRGY-PIEQLAEKS--DY-----L-ETCYLLLNG-EL--P-TAAQK
7 TNGNNNN--LE--FAELKIRGKLFKLPILKASIGKDV-I-DISRVSAEADYF--T--YDPGFMSTASCQ-STITYIDGDKGILWYRGY-DIKDLAEKS--DF-----L-EVAYLMIYG-EL--P-SSDQY
8 ----------------------------------------------------PETEEISKGLEDVNI-KWTRLTTIDGNKGILRYGGY-SVEDIIASGAQDEE----I-QYLFLYGN---L--P-TEQEL
                                                              G                         G

  131    140       150       160       170       180       190       200       210       220       230       240       250       260
  SWLSKEWAKRAA-LPSHVVTMLDNFPTNLHPMSQLSAAITALNSESNFARAYAEGIHRTKYWELIYEDCMDLIAKLPCVAAKIYRNLYREGSSIGAIDSKLDWSHNFTNMLGYTDAQFTELMRLYLTIHS
  KLKHCRKTWNRAAVSDYVYNAIDALPSTAHPMTQFASGVMALQVQSEFQKAYENGIHKSKFWEPTYEDCLNLIARVPVVAAYVYRRMYKNGDSIPSDKSLD-YGANFSHMLGFDDERLKELMRLTSPSTV
  KALSADLAARSE-IPEHVIQLLDSLPKDLHPMAQFSIAVTALESESKFAKAYAQGVSKKEYWSYTFEDSLDLLGKLPVIASKIYRNVFKDGK-ITSTDPNADYGKNLAQLLGYENKDFIDLMRLYLTIHS
  DEFKTTVTRHTM-IHEQITRLFHAFRRDSHPMAVMCGITGALAAFYHDSLDVNNPRHREIAAFRLLSKMPIMAAMCYKYSIGQPFVYPRN-D-L-S------YAGNFLNMM-FSTPCEPYEVNPILERAM
  VEFDAKVRAHTM-VHDQVSRFFNGFRRDAHPMAIMVGVVGALSAFYHNNLDIEDINHREITAIRLIAKIPTLAAWSYKYTVGQPFIYPRN-D-L-N------YAENFLHMM-FATPADRDYKVNPVLARA
  EQFVGTIKNHTM-VHEQLKTFFNGFRRDAHPMAVMCGVIGALSAFYHDSLDITNPKHREVSAHRLIAKMPTIAAMVYKYSKGEPMMYPRN-D-L-N------YAENFLHMM-FNTPCETKPISPVLAKAM
  CNFTKKVAHHSL-VNERLHYLFQTFCSSSHPMAIMLAAVGSLSAFYPDLLNFNETDY-ELTAIRMIAKIPTIAAMSYKYSIGQPFIYPDN-S-L-D------FTENFLHMM-FATPCTKYKVNPIIKNAL
  RKYKETVQKGYK-IPDFVINAIRQLPRESDAVAMQMAAVAAMAASETKFKWNKDTD-RDVAAEMIGRMSAITVNVYRHIMNMPAELPKPS-D---S------YAESFLNAA-FGRKATKEEIDAMNTALI

  261    270       280       290       300       310       320       330       340       350       360       370       380       390
  ---------DHEGGNVSAHTSHLVGSALSDPYLSFAAAMNGLAGPLHGLANQEVLVWLTQLQKEVGKDVSDEKLRDYIWNTLNSGRVVPGYGHAVLRKTDPRYTCQREFALKHLPH----D--PMFKLVA
  M--------HEGGNVSAHTGHLVGSALSDPYLSFAAALNGLAGPLHGLANQEVLLWIKSVVEECGEDISKEQLKEYVWKTLNSGKVIPGYGHGVLRNTDPRYVCQREFALKHHPD----D--PLFQCCK
  ---------DHEGGNVSAHTTHLVGSALSSPYLSLAAGLNGLAGPLHGRANQEVLEWLFKLREEVKGDYSKETIEKYLWDTLNAGRVVPGYGHAVLRKTDPRYTAQREFALKHFPDY---E---LFKLVS
  D-RILILHADHEQ-NASTSTVRTAGSSGANPFACIAAGIASLWGPAHGGANEAALKMLEEISS-VK-HIPEFFRRAKDKNDSFR--LM-GFGHRVYKNYDPRATVMRETCHEVLKELGTKDD--LLEVAM
  MDRIFTLHADHEQ-NASTSTVRLAGSTGANPYACISAGISALWGPAHGGANEAVLKMLDEIGS-VE-NVAEFMEKVKRKEVKLM-----GFGHRVYKNFDPRAKVMKQTCDEVLEALGIND--PQLALAM
  D-RIFILHADHEQ-NASTSTVRLAGSSGANPFACIASGIAALWGPAHGGANEAVLRMLDEIGD-VS-NIDKFVEKAKDKNDPFK--LM-GFGHRVYKNFDPRAKVMKQTCDEVLQELGIND--PQLELAM
  N-KIFILHADHEQ-NASTSTVRIAGSSGANPFACISTGIASLWGPAHGGANEAVINMLKEIGSSEN--IPKYVAKAKDKNDPFR--LM-GFGHRVYKSYDPRAAVLKETCKEVLNELGQLDNNPLLQIAI
  L---YT---DHEVP-ASTTAGLVAVSTLSDMYSGITAALAALKGPLHGGAAEAAIAQFDEIKDPAM--VEKWFNDNIINGKKRL---M-GFGHRVYKTYDPRAKIFKGIAEKLSSKKPEVHK--VYEIAT
            HE    S   .    S               L GP HG A                                       G GH V    DPR

  391    400       410       420       430       440       450       460       470       400       490       500       510       520
  QLYKIVPNVLLEQGKAKNPWPNVDAHSGVLLQYYGM-TEMNYYTVLFGVSRALGVLAQLIWSRALGFP-LERPKSMSTDGLIKLV-D---SK
  LMKLASCLTELESEEP---WPNVDAHSGVLLNHYGL-TEARYYTVLFGVSRSLGICSQLIWDRELLLA-LERPKSV-TMDWLEAHCKK-ASSA
  TIYEVAPGVLTKHGKTKNPWPNVDSHSGVLLQYYGL-TEASFYTVLFGVARAIGVLPQLIIDRAVGAP-IERPKSFSTEKYKELVKK-IESKN
  ELENIALNDPYFIEK-KL-YPNVDFYSGIILKAMGIPSSM--FTVIFAMARTVGWIAHWSEMHSDGMK-IARPRQLYIGYEKRDFKSDIK-R
  ELERIALNDPYFVER-KL-YPNVDFYSGIILKAIGIPTEM--FTVIFALARTVGWISHWLEMHSGPYK-IGRPRQLYTGEVQRDI-K----R
  KLEEIARHDPYFVER-NL-YPNVDFYSGIILKAIGIPTSM--FTVIFALARTVGWISHNQEMLSGPYK-IGRPRQLYTGHTQRDFTA-LKDRG
  ELEALALKDEYFIER-KL-YPNVDFYSGIIYKAMGIPSQM--FTVLFAIARTVGWMAQWKEMHEDPEQKISRPRQLYTGYVHREYKC-IVERK
  KLEDFGIKAFGSKGI----YPNTDYFSGIVYMSIGFPLRNNIYTALFALSRVTGWQAHFIEYVEEQQRLI-RPRAVYVGPAERKYVP-IAERK
                      PN D  SG      G        T  F   R  G                 RP
```

1. Pig Heart
2. *A.thaliana*
3. *S.cerevisiae*
4. *E.coli*
5. *A.anitritum*
6. *P.aeruginosa*
7. *R.prowasekii*
8. *T.acidophilum*

FINAL DISCUSSION

The question still remains, therefore, as to the nature of this mutant CS from K114r4. There exist several possible explanations.

The first is that the protein is a second species of CS. There are many instances emerging from mutation studies of pairs or groups of isoenzymes in *E.coli* that catalyse the same reaction but can be distinguished from each other by their kinetic and molecular properties. The genes for many of these isoenzymes have been mapped showing the homologous enzymes to be products of different genes (Table 8.1). This is in contrast, for example, to the isoenzymes of alkaline phosphatase that are encoded by only one gene (Garen & Garen, 1963) and are formed by post-translational proteolysis (Schlesinger *et al.*, 1975; Nakata *et al.*, 1982). In instances where isoenzymes are thought to be a result of gene duplication there should be a high level of homology between their sequences. Indeed, the three isoenzymes of DAHP synthetase [EC 4.1.2.15] have a high level of homology at the N-terminal amino acid sequence (Shultz *et al.*, 1981) and comparison of the nucleotide sequence of two of the three genes (*aro*H and *aro*G) gave rise to high homology (Davies & Davidson, 1982) indicating that the two genes have arisen from a common ancestor by divergent evolution. However, there are many instances where, as in this study, enzymes catalyse the same reaction but are immunologically unrelated (Kotlarz & Buc, 1977; Claiborne *et al.*, 1979; Richarme, 1989). This could be because genetic changes in the course of evolution have been such that many antigenic sites have been lost whilst the active site has remained intact. Or it could be that the observed similar functions are a consequence of convergent evolution from separate ancestral genes. It is interesting to note that one of the enzymes from an enzyme pair frequently resembles the eukaryotic form of the enzyme (Waygood & Sanwal, 1974; Daldal, 1984;

ENZYME	GENE	MAP	REFERENCE
ACETOLACTATE	*ilv*B	83	Friden *et al.*,1985
SYNTHASE	*ilv*HI	2	Squires *et al*, 1983
(valine sensitive)			
ASPARAGINE	*asn*A	84	Buhk and Messer, 1983
SYNTHETASE	*asn*B	16	Felton *et al*, 1980
			Plumbridge, 1984
CATALASE	*kat*G	89.2	Loewen *et al*, 1985
	*kat*E	37.8	Loewen, 1984
DAHP SYNTHASE	*aro*F	57	Hudson & Davidson, 1984
	*aro*G	17	" " "
	*aro*H	37	Bachmann, 1983
β-KETOACYL-ACP	*fab*B	50	Garwin *et al.*, 1980a
	*fab*F	24.5	Garwin *et al.*, 1980b
LIPOAMIDE DH	*lpd*	3	Guest *et al*, 1983
	?	?	Richarme, 1989
NITRATE REDUCTASE	*nar*ZYWV	33	Bonnefoy *et al.*, 1987
	*nar*GHJI	27	Bachmann, 1991
ORNITHINE	*arg*F	7	Sens *et al.*, 1977
TRANSCARBAMYLASE	*arg*I	97	Roof *et al*, 1982
PGP-	*pgp*A	10	Icho & Raetz, 1983
DEPHOSPHORYLASE	*pgp*B	28	" " "
PHOSPHOFRUCTOKINASE	*pfk*A	88	Morrissy & Fraenkel, 1969
	*pfk*B	38	Morrissy & Fraenkel, 1972
PYRUVATE KINASE	*pyk*A		
	*pyk*F	37	Garrido-Pertierra & Cooper, 1983
SHIKIMATE KINASE	?		
	*aro*L	9	DeFeyter & Pittard, 1986
tRNA Species	various		Komine *et al*, 1990
EF-Tu	*tuf*A	77.5	Bachmann & Low, 1980
	*tuf*B	89	" " "

Table 8.1 Isoenzymes

Millar *et al.*, 1986; Alefounder *et al.*, 1989; Scofield *et al.*, 1990) as does the CS observed in this study.

If there does exist a second structural gene for CS it is most likely to have arisen by way of tandem duplication of the *glt*A gene. Gene duplication has been implicated as an important factor in evolution and bacterial adaptation (Lewis, 1951; Ohno, 1970; Zipkas & Riley, 1975) and has been proposed as a mechanism for the evolution of biosynthetic pathways (Véron *et al.*, 1972; Truffa-Bachi *et al.*, 1975; Belfaiza *et al.*, 1986).

Danson *et al.* (1979) performed transduction experiments with P1 phage using their mutant CS*E.coli* as donor and the strain K2-1-4 (F^-, *gal*$^-$, *glt*A$^-$) as the recipient. These experiments gave a co-transduction frequency of the *gal* and *glt*A genes of 22%. This positions the mutant CS gene 36.2 kb (Wu, 1966) or 0.9 min on the genome away from the *gal* gene. However, their transduction experiments were non-directional and thus there are two possible locations for the mutant CS gene. As the *glt*A gene maps 0.8 min away from the *gal* gene (Ashworth *et al.*, 1965) the mutant gene could be a modified form of the *glt*A gene (as suggested by Danson *et al.*, 1979) and would, therefore, have to be a different mutant from that in this study. This is unlikely because the two mutants display more or less identical properties. The second possibility is that the mutant CS is a product of a gene 0.9 min *upstream* from the *gal* gene and could, therefore, have resulted from tandem duplication of the *glt*A gene followed by mutations arising in a change in physical properties of the protein without loss of function. Examination of the genes currently mapped in that region (Fig. 8.1) does not shed any light on the identity of the mutant CS.

Multiple forms of an enzyme may be required to catalyse the same reaction but under different metabolic conditions (e.g isoenzymes of

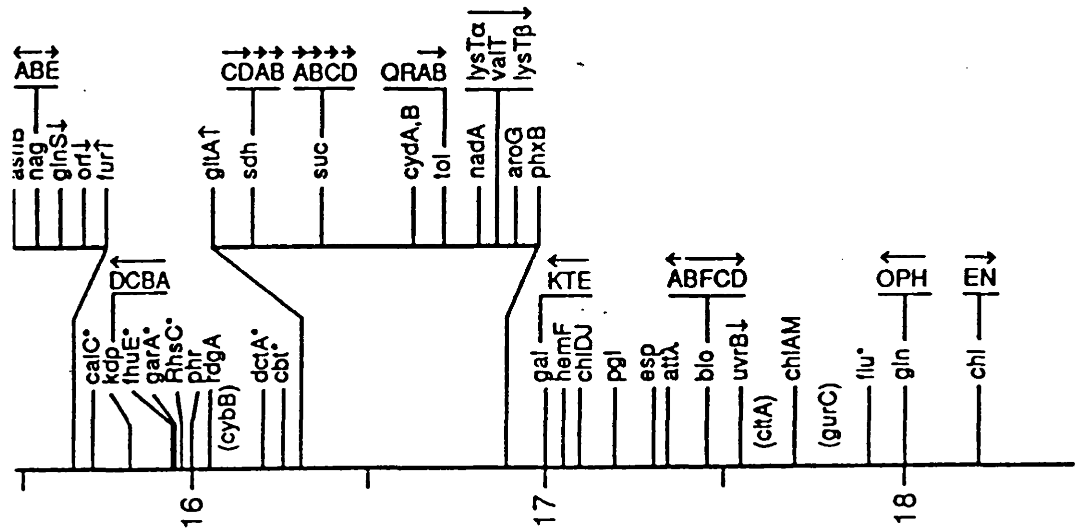


Fig. 8.1. Gene Map of the Region Flanking *glt*A

pyruvate kinase, Malcovati & Kornberg, 1969), in different places in the same cell (e.g isoenzymes of asparaginase, Cedar & Schwartz, 1967) or at different stages of differentiation (e.g isoenzymes of catalase, Loewen *et al.*, 1985a) or to increase the cellular amount of the enzyme via the gene dosage effect (Ikemura & Ozeki, 1983). As yet, there is no evidence that two forms of CS co-exist in wild type *E.coli.* However, under normal conditions, the mutant CS has low activity which may be masked by the higher activity of the wild type enzyme. Relatively little is known about the mechanism controlling the transcription of the *glt*A gene, although it is known that it is induced by aerobic conditions and growth on acetate and repressed by glucose and anaerobiosis and that its synthesis is inversely proportional to growth rate (Gray *et al.,*, 1966; Smith & Neidhardt, 1983). Could it be that a second gene exists that is induced under conditions different to those for the *glt*A gene and has been mutated such that it is now constituitively produced? The mutant protein appears to be under different control as its activity is severely depressed in the presence of glucose (this study and Else, 1986). In addition, it has been suggested that many isoenzyme exist to catalyse different pathways (Malcovati & Kornberg, 1969; Garwin *et al.*, 1980;Weiss & Edwards, 1980; Stribling & Perham,1973; Loewen *et al.*, 1983; Guest *et al.* 1985) and it may be that the mutant CS normally catalyses the glyoxylate pathway.

A second possibility is that the mutant CS may somehow be related to another enzyme. CS catalyses the condensation of acetyl-CoA with OAA (an α-keto acid). Several other enzymes,involved in amino acid biosynthesis catalyse a similar reaction (Fig. 8.2) and it has been proposed that these may all share a common ancestor (Jensen, 1976). As the catalytic mechanism is most probably the same in each case, one could envisage

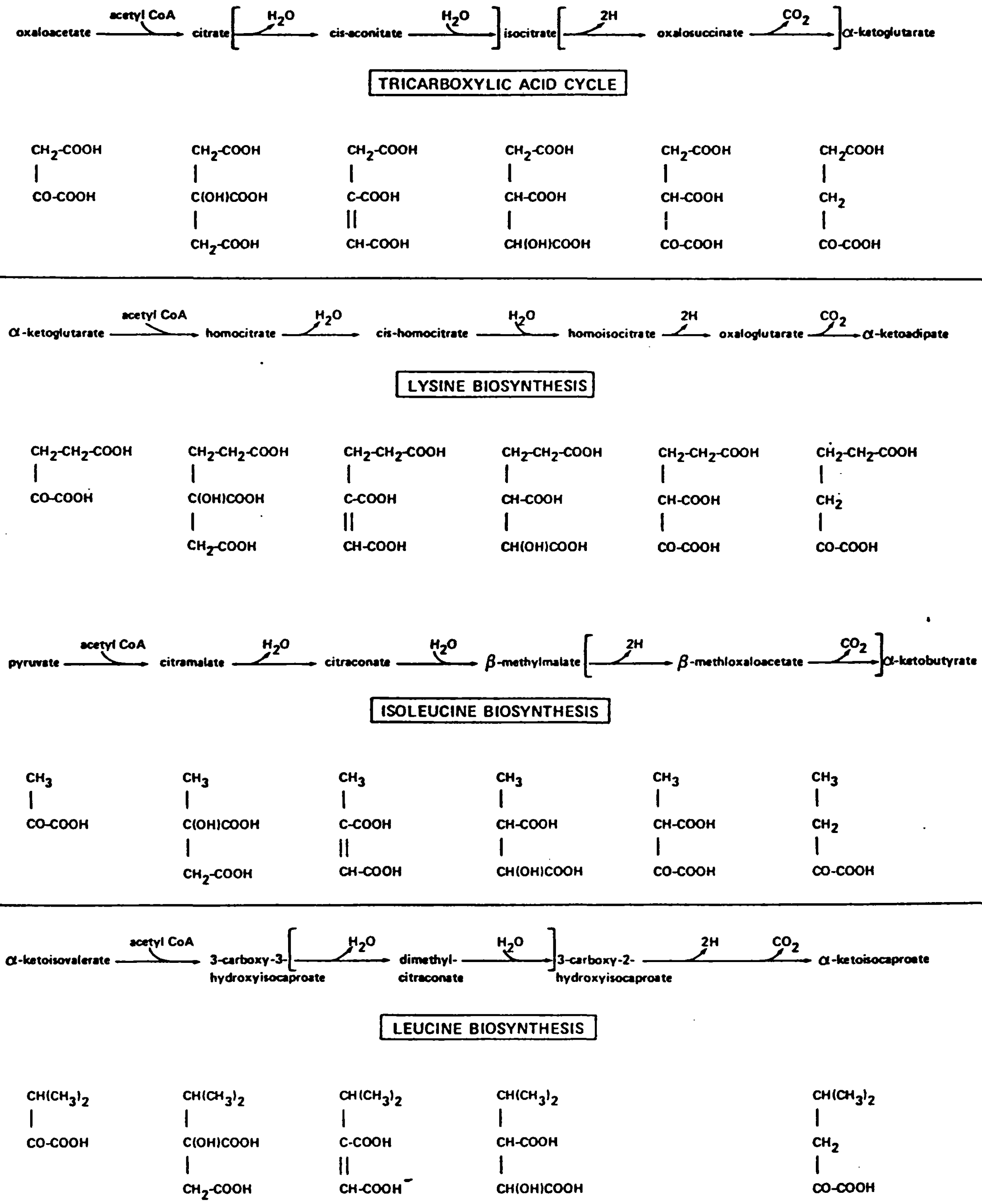


Fig. 8.2. Analogous Reaction Sequences

that in their native state or by minor modifications this group of enzymes may be able to utilise different α-keto acids, albeit to a lesser extent. Indeed, the activity of the mutant CS is considerably less than that of the wild type enzyme. Several studies have shown that the ability to metabolise novel carbon sources is achieved by 'recruiting' existing proteins that catalyse reactions similar to those needed (LeBlanc & Mortlock, 1972;Cocks *et al.*, 1974; Stevens & Wu, 1976; Wu, 1976a,b). Minor mutations that may effect the regulation and/or structure of those particular enzymes can then lead to adequate utilisation of the new substrate. For example, *E.coli* lacks the ability to grow on D-arabinose but by mutation that alters the specificity of induction of enzymes of the pathway of L-fucose catabolism *E.coli* can utilise arabinose as sole carbon source (LeBlanc & Mortlock, 1972).In such a mutant D-arabinose was able to induce the fucose enzymes and the broad specificity of these enzymes then permitted poor but adequate growth on D-arabinose. Similar exploitation of substrate ambiguity has been demonstrated in *E.coli* to acquire other new metabolic functions (Hegemen & Rosenberg, 1970). In some mutants the enzymes recruited had known functions in *E.coli* whereas others gained enzymatic activity whose normal functions were unknown.

Site directed mutagenesis studies have also shown that substrate specificity can be dramatically changed by minor mutations around the active site. Insertion of a 14 amino acid oligopeptide into the adenylate synthesis domain of *E.coli* methionyl-tRNA synthetase produced a mutant that showed adenylate synthesis activity (Starzyk *et al.*, 1989). In addition, by making just three base substitutions the specificity of NAD-dependent lactate dehydrogenase from *Bacillus stearothermophillus* was shifted from lactate to malate (Wilks *et al.* ,1988).

A third possibility is that the mutant CS is a product of a normally silent gene. The most well studied example of such a recruitment of a silent unexpressed gene is the *ebg* (evolved β-galactosidase) system (Campbell *et al.*, 1973; Hall & Hartl, 1974; Hall & Hartl, 1975). The wild type *ebg* operon does not permit utilisation of lactose or other β-galactoside sugars (Hartl & Hall, 1974). However, in *E.coli lacZ* (β-galactosidase) deletion strains, spontaneous mutations occur in the *ebg* operon that allow utilisation of lactose (Campbell *et al.*, 1973, Hall & Hartl, 1974; Hall, 1977). Other mutants of *ebg* have been isolated that allow utilisation of lactulose, galactosylarabinose or lactobionic acid (Hall, 1978; Hall & Zuzel, 1980). The *ebg* operon is distinct from *lacZ* and the *ebg* enzyme is immunologically unrelated to the *lacZ* gene product (Campbell *et al.*, 1973; Arraj & Campbell, 1975). DNA sequence analysis has revealed that only a single base change in the *ebg* repressor gene and one to three base changes in the *ebg* structural gene were responsible for activation and substrate specificity (Stokes *et al.*, 1985; Stokes & Hall, 1985; Hall *et al.*, 1989). Despite their functional similarity the *ebg* β-galactosidase displays only 33.7% overall homology with *E.coli* β-galactosidase, although the active site residues are highly conserved (Hall, 1989).

Thus there is no simple answer to the possible origin of the mutant protein. Further work is obviously necessary in order to identify the protein and its metabolic function. As the N-terminal sequence of the protein has been determined it will be possible to construct an oligonucleotide probe with which the gene may be identified from restriction digests of *E.coli* chromosomal DNA and cloned.

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